RECOMBINANT CELL LINE AND SCREENING METHOD 09/674876
FOR IDENTIFYING AGENTS WHICH REGULATE APOPTOSIS AND
TUMOR SUPPRESSION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C
119(e) from U.S. Provisional Application Nos. 60/084,664
filed May 7, 1998, 60/091,391, filed July 1, 1998,
60/092,871 filed July 15, 1998 and 60/107,689 filed

5   November 9, 1998, the disclosure of each being
incorporated by reference herein.

Pursuant to 35 U.S.C. §202(c) it is acknowledged that
the U.S. Government has certain rights in the invention
described herein, which was made in part with funds from

10   the National Institutes of Health, Grant Number CA-60088.

FIELD OF THE INVENTION

The present invention relates to the identification
of anti-neoplastic agents. Specifically, the invention

15   provides novel cell-based assays and reagents for
screening compounds involved in the regulation of
apoptosis and tumor suppression.

BACKGROUND OF THE INVENTION

20   Several publications are referenced in this
application by author name and year of publication in
parentheses in order to more fully describe the state of
the art to which this invention pertains. Full
citations for these references are found at the end of

25   the specification. The disclosure of each of these

publications is incorporated by reference herein.

Apoptosis is a genetically controlled pathway of programmed cell death. The hallmarks of apoptosis are cytoplasmic boiling, chromatin condensation, and DNA fragmentation. Apoptosis plays a major role in development and disease. Regulation of cell death is essential for normal development and is an important defense against viral infection and the emergence of cancer. Excessive cell death can lead to impaired development and degenerative diseases, whereas too little cell death can lead to cancer and persistent and sustained viral infection.

Tumor suppressor genes are a class of genes involved in different aspects of normal control of cellular growth and division. Inactivation of tumor suppressor genes, usually by genetic means, contributes to tumor development. The first evidence for the existence of such genes came from the observation that fusion of normal cells with tumor cells resulted in hybrids which were nontumorigenic. However, with the loss of chromosomes, which occurs naturally when hybrid cells are grown in culture, the tumorigenic phenotype was sometimes restored. Furthermore, the introduction of normal chromosomes into tumorigenic hybrids can cause reversal to the nontumorigenic phenotype. Thus expression of genes from the normal parent suppresses tumorigenicity.

As the normal functioning of tumor suppressor genes suppresses tumorigenicity, regions of chromosomes which are lost in tumors are likely to carry tumor suppressor genes. Karyotype analysis of a wide variety of tumor types has shown that certain chromosomal deletions occur with a high frequency and in a tumor specific manner,

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thus implicating the chromosomal region as a likely location of tumor suppressor genes.

Apoptosis is controlled through the expression of an increasing number of genes conserved in nematodes through mammals and viruses. Some gene products are activators of apoptosis, whereas others are inhibitors. Tumor suppressor genes are also widely conserved. The characterization of the function of these gene products will facilitate elucidation of the molecular mechanisms which regulate cell growth at the biochemical level. This knowledge in turn, facilitates the identification and characterization of agents suitable for regulating apoptotic cell death and tumor suppression which may be used to advantage in the treatment of malignancy and other disorders characterized by aberrant cell growth.

SUMMARY OF THE INVENTION

In accordance with the present invention, it has been discovered that inhibition of the transcriptional coactivator p300 induces p53 accumulation resulting apoptotic cell death. p300 is required to transactivate the p53 inhibitor mdm2. Failure of p300 to transactivate mdm2 results in accumulation of p53 to high levels causing apoptosis, a desirable characteristic of anti-cancer therapeutics. The present invention is directed to the identification of p300 transactivating inhibitors which act to induce p53 accumulation through the failure to coactivate mdm2. Such agents should be effective to induce apoptotic cell death for the treatment of proliferative disorders associated aberrant cell growth.

The present invention provides novel cell lines and methods for identifying agents that regulate apoptosis.

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According to one aspect of the invention a recombinant cell line is provided for identifying and characterizing p300 inhibitors which regulate apoptosis. An exemplary cell line of the invention comprises a) a first plasmid expressing a p300 responsive promoter operably linked to a first reporter gene; b) a second plasmid expressing a non p300 responsive promoter operably linked to a second reporter gene; and c) a third plasmid expressing a selectable marker gene. The cell line may optionally include a plasmid encoding wild -type p300 to augment endogenously expressed p300 protein levels.

In another embodiment of the invention, a screening method is provided using the cell line of the invention to identify therapeutic agents that regulate apoptosis. The screening method entails determining whether a potential therapeutic reagent inhibits p300 activity thereby inducing apoptosis. One such method is performed by contacting the recombinant cells of the invention with the therapeutic agent. The recombinant cells include a first plasmid expressing a p300 responsive promoter operably linked to a first reporter gene, a second plasmid expressing a non p300 responsive promoter operably linked to a second reporter gene; and a third plasmid expressing a selectable marker gene. Following administration of the reagent repression of the p300 responsive reporter and repression of the non-p300 responsive reporter gene by the therapeutic reagent would be assessed via the levels of reporter gene activity. Repression or the p300 responsive reporter gene and not the non-responsive p300 reporter gene indicates that the compound inhibits p300 transactivation and thereby may be efficacious in inducing apoptosis in target cells. In an alternative

embodiment, plasmids may be constructed which contain a
plurality of the above described gene constructs.

In yet another embodiment of the invention, a Bcl-2
associated transcription factor (referred to herein as
BP-1 and btf) having the sequence of SEQ ID NO: 1 has
been identified which interacts with E1B 19K as well as
with anti-apoptotic family members, Bcl-2 and Bcl-$_{XL,}$ but
not the proapoptotic protein, Bax. Btf protein has a
sequence of SEQ ID NO:2, binds DNA in vitro and
represses transcription in reporter assays. The Btf
gene has been localized to chromosome 6 (6q22-23), a
region that is deleted in certain cancers providing
support for Btf's role as a tumor suppressor. Btf is
also active in a unique pathway by which Bcl-2 regulates
apoptosis.

The availability of the DNA sequence encoding btf
facilitates the production of large amounts of btf
protein in suitable expression systems. The Btf protein
so generated may be used to advantage in the production
of antibodies or antibody fragments that may be used to
detect Btf protein in clinical samples.

Elucidation of the interaction between Bcl-2 and
Btf facilitates the identification and characterization
of novel therapeutic agents that specifically disrupt
the binding the Bcl-2 and Btf, while not affecting Bcl-2
binding to other proteins. Agents that disrupt binding
between Bcl-2 and Btf may be assessed in cell lines
which co-express the two proteins. Such agents may
comprise peptides or other small molecule inhibitors
which act competitively to inhibit the binding between
Bcl-2 and Btf, there by effectuating cell death in a
variety of tumors.

In accordance with the present invention, methods

are provided for screening tumor samples for deletion of
the Btf gene and/or protein. Such methods may be either
immunological and employ Btf specific antibodies to
detect the presence or absence of btf protein

5   expression. Alternatively, the methods may be
genetically based. Btf specific oligonucleotides may be
employed to assess the sample for the presence or
absence of btf encoding nucleic acids.   In patients
having a btf deletion, the Btf gene may be reintroduced

10   via genetic engineering methods.

The cell lines and screening methods of the present
invention provide a unique and advantageous testing
system for evaluating reagents that regulate apoptosis.
Such agents may then be used to advantage to treat

15   pathological conditions related to aberrant cell growth,
including cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

20   Figures 1A and 1B show the results of Western blot
analysis of p53, Mdm2, Bax, and p21^{WAF1} in p53A and LTR.1A
cells after incubation at the nonpermissive (37.5°C) and
permissive (32°C) temperature at day 0 (lanes 1 and 6),
day 1 (lanes 2 and 7), day 2 (lanes 3 and 8), day 3

25   (lanes 4 and 9) and day 5 (lanes 5 and 10).

Figure 2 shows the results obtained from indirect
immunofluorescence of Mdm2 in p53A (top panel) and
LTR.1A (bottom panel) cells after incubation at 32°C

30   from day 0 to day 3.

Figures 3A and 3B are a pair of graphs showing
cell viability as demonstrated by trypan blue staining

of p53A and LTR.1A cells at the nonpermissive (37.5°C)
and permissive (32°C) temperature from day 0 to day 3.

Figure 4 depicts a series of micrographs showing
light microscopy analysis of p53A and LTR.1A cells
incubated at 32°C from day 0 to day 3 to detect
apoptotic cells.

Figures 5A and 5B show the results of Northern blot
analysis of *mdm2* expression of p53A cells incubated at
32°C for the indicated times. Fig. 5A, autoradiograph
of blot; Fig. 5A ethidium stained gel showing loading
and integrity of RNA.

Figure 6 is an autoradiograph showing the results
of Western blot analysis of ectopically expressed HA-
tagged p300, endogenous Mdm2, and actin in p53A cells
transiently transfected by electroporation with pCMVβ
alone (lane 1 - 12 μg and lane 2 - 24 μg), pCMVβp300
(p300 wild-type) (lane 3 - 12 μg and lane 4 - 24 μg) or
p300Δ30 (p300 mutant) (lane 5 - 12 μg and lane 6 - 24
μg).

Figure 7 shows the results of indirect
immunofluorescence of HA-tagged p300 and endogenous Mdm2
in p53A cells transiently transfected with 24 μg of
vector alone, wild-type p300 and mutant p300 (arrows
indicate cells with ectopic p300 expression and up-
regulation of Mdm2).

Figure 8 is a bar graph showing cell viability, as
measured by trypan blue staining, of p53A cells

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transiently transfected with vector alone, wild-type p300 or mutant p300.

Figure 9 is a graph showing the percentage of apoptotic cells that are positive for ectopic expression of a control lamin fragment, p300 wild-type and p300 mutant determined by cell morphology using indirect immunofluorescence in p53A cells transiently transfected with 24 μg of pCEP4-LA(1-406), pCMVβp300 or pCMVβp300Δ30 plasmid DNAs.

Figure 10 shows the results of Western Blot analysis of EIA, Mdm2 and actin in LTR.1A cells transiently transfected with 10 μg of vector alone (lane 1), 12S E1A (lane 2), 12SRG2 (lane 3), or 12S.YH47.928 (lane 4).

Figure 11 shows cell viability, as measured by trypan blue exclusion, of LTR.1A cells transiently transfected with vector alone, 12S E1A, 12S.RG2 or 12S.YH47.928.

Figure 12 shows Western blot analysis of p53A cells transiently transfected with 5 and 10 μg of pCMV vector alone, mdm2 wild-type (pCOC-X$_2$) or mdm2 mutant (pCOC-ΔXM) plasmid DNAs.

Figure 13 is a graph showing cell viability as assessed by trypan blue staining of p53A cells transiently transfected with mdm2 expression plasmids incubated at 32°.

Figure 14 is a graph showing the percentage of

apoptotic cells that were positive for ectopic
expression of a control lamin fragment, Mdm2 wild-type,
and Mdm2 mutant determined by cell morphology using
indirect immunofluorescence in p53A cells transiently

5   transfected with 10 μg of pCEP4-LA(1-406), pCOC-X$_2$ (wild-
type Mdm2) or pCOC-ΔXM (truncated Mdm2) plasmid DNAs.

       Figures 15A, 15B and 15C show the results of
Western blot analysis of p53, Mdm2, bax, and p21Waf1 in

10   p53A cells stably expressing PSVneo (p53An1) E1B19K
(19K1) or Bcl-2 (4B).

       Figure 16 shows Western blot analysis of Mdm2, Bcl-
2 E1B 19K and actin in p53An1 cells transfected

15   transiently in pCNDA3 alone, (lane 1); pCMV19K (lane 2)
and pCNA3myc-tagged bcl-2, (lane 3).

       Figure 17 shows the results of Northern blot
analysis of Mdm2 expression using 30 μg of cytoplasmic

20   RNA of 19K1 cells incubated at 32°C for the indicated
time intervals.

       Figure 18 is a schematic diagram illustrating the
regulation of cell death in p53A, LTR.1A and 19K1/4B

25   cells.

       Figures 19A and 19B are growth profiles of
transformed yeast cells demonstrating that BP-1
interacts with E1B 19K in yeast. The yeast two-hybrid

30   assay was utilized to demonstrate binding between BP-1
and E1B 19K. Growth in the presence of histidine
indicates that both plasmids can be expressed in yeast,
and growth in the absence of histidine demonstrates an

interaction between the two proteins. Apc-2 represents
an irrelevant hydrophobic protein used as a negative
control. The specificity of the interaction between BP-
1 and E1B 19K was tested using related proteins (Bcl-2,

5   Bcl-x_L, and Bax) as well as missense mutants of E1B 19K
(pm7, pm51, pm87, and pm102, Fig. 19A. Fig. 19B shows
growth profiles which reveal the minimal regions of E1B
19K required for interaction with BP-1 were mapped using
a series of deletion mutants. Figure 19C is a schematic

10   representation of E1B 19K showing the missense and
deletion mutants tested in the two-hybrid assay.
Regions I and III indicate the locations of the Bcl-2
homology domains, BH1 and BH3. E1B 19K does not contain
recognizable BH2 or BH4 domains.

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Figures 20A and 20B are autoradiographs showing the
results of Northern blot analysis performed to determine
btf expression in various tissues (Fig. 20A), and cancer
cell lines (Fig. 20B). Two transcripts were observed at

20   5 kB (*btf*$_L$) and 3 kB (*btf*$_s$). These transcripts appeared
to be ubiquitously expressed, except that *btf* was not
detected in Raji cells derived from Burkitt's lymphoma.
Lower panels depict β-actin expression to assess the
quality of the RNA and to control for loading

25   efficiency.

Figure 21 depicts the amino acid sequence of Btf.
Btf$_L$, identified in the GenBank database (accession #
D79986) is predicted to encode a 918 amino acid protein

30   shown here. Btf$_s$, which was obtained in the λ-screen,
contains the complete Btf$_L$ sequence except that it is
missing 49 amino acids between residues 797 and 846
(bold) present in Btf$_L$ and BP-1. The underlined regions

represent the positions of nuclear localization
sequences.

Figure 22 is a schematic representation of the Btf
variants and deletion mutants used to characterize Btf
function. The gray and black boxes represent the
locations of putative bZIP and Myb-DNA binding domains,
respectively. BP-1 (N384), identified in the yeast two-
hybrid screen for E1B 19K binding proteins, contains
residues 384-918 of Btf_L. Deletion mutants of Btf_s, N552
and C210 were used in transcriptional reporter assays to
determine the regions of Btf_s that contribute to
transcriptional repression.

Figure 23 is an autoradiograph showing ^{35}[S]-
methione labeled *in vitro* translated Myc-Btf_s and E1B 19K
prepared using the TNT T7 reticolocyte lysate system and
incubated with native DNA-cellulose in NETN buffer for 2
hrs. The data show that Btf_s binds to DNA-cellulose *in
vitro*. Samples were washed five times in NETN and the
proteins were resolved by 17% SDS-PAGE.
Immunoprecipitated proteins (lanes 1 and 2) were
analyzed to confirm the presence of the *in vitro*
translated products.

Figure 24 is an autoradiograph showing *in vitro*
interactions betwen Btf_s and anti-apoptotic Bcl-2 family
members. ^{35}[S]-labeled *in vitro* translated Myc-Btf_s or
Myc-Bax was combined with V5/His-E1B 19K, Bcl-2, Flag-
Bcl-x_L, or luciferase prepared using the TNT T7
reticolocyte lysate system. The proteins were
immunoprecipitated with anti-Myc monoclonal antibody in
NETN buffer for 1.5 hrs followed by protein A-sepharose

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for 0.5 hr.   Samples were washed in NETN buffer,
resolved by 14% SDS-PAGE and visualized by
autoradiography. In addition, 1 μl of each of
translation reaction was analyzed to verify that equal

5   amounts of proteins were used in the binding assay.

Figure 25 is a series of micrographs showing that
the nuclear subcellular localization of Btf_s is altered
by E1B 19K, Bcl-2 and $Bcl-x_L$.   HeLa cells were

10   transfected with expression plasmids pcDNA3-Myc- Btf_s
alone and with pCMV E1B 19K or pcDNA3-Bcl-2 as
indicated.   Cells were fixed 24 hours post-transfection
and double stained against Myc and E1B 19K or Bcl-2. All
cells were photographed with an original magnification

15   of 1000X.

Figure 26 is a graph showing the percent of nuclear
Btf_s expression (Btf_s /total Btf_s) in HeLa cells
expressing $Bcl-x_L$ transfected with pcDNA3-Myc-Btf_s and

20   stained 24 hours post-transfection against Myc.

Figure 27 is a graph showing the results of a
reporter assay demonstrating that Btf_s is a transcription
repressor and is inhibited by Bcl-2-like proteins.   HeLa

25   cells were transfected with 2.5 μg of the reporter
construct (luciferase construct containing GAL-4 DNA-
binding sites within its promoter), 2.5 μg of GAL-4 DNA-
binding domain fusion genes (pml- Btf_s,Δ-N552, and Δ-C210
or empty pml vector control), and 2.5 μg of bcl-2 family

30   gene (pCMV-E1B 19K, pcDNA3-Bcl-2, or pcDNA3-Flag-$Bcl-x_L$,
or empty pcDNA3 vector control).   Cells were harvested
24 hours post-transfection and the luciferase activity
was measured in a scintillation counter using the

luciferase substrate, luciferin. Values were normalized
for protein concentrations measured by a Bradford assay
and graphed as a percent of the negative control (empty
pml vector). The experiment was repeated six times and
the high and low values for each sample were dropped.
Bars indicate standard deviation, n=4.

        Figures 28A and 28B are a pair of graphs showing
that Btf$_s$ inhibits transformation by E1A and E1B 19K
(Fig. 28A) or p53DD (Fig. 28B). Primary baby rat kidney
(BRK) cells were transfected with carrier DNA along with
linearized test DNA (15 μg pCMV-E1A, 15 μg pCMV-E1B 19K
or pCMV-p53DD, and 45 μg pcDNA3-Myc- Btf$_s$). DNA
concentrations were kept constant using appropriate
empty vectors. The cells were cultured for 3 to 4 weeks
and then stained with Giemsa. Foci were counted from
four dishes per condition. Bars indicate standard
deviation, n=4.

        Figure 29 shows a representative FACS scan from
Table 1 at 72 hours post-transfection of pIRES-EGFP-Btf$_s$
and the empty pIRES-EGFP vector into HeLa cells   The
data reveal that Btf$_s$-mediated cell death occurs by
apoptosis. Cell cycle kinetics from propidium iodide
staining are shown for the total cell population as well
as the EGFP-positive cells.

        Figure 30 is a series of micrographs showing
transfected cells incubated with Hoechst dye 72 hours
post-transfection to visualize the DNA. Cells
transfected with pIRES-EGFP-Btf$_s$ (right side) were
compared to control pIRES-EGFP (left side). Arrows for
each set correspond to the same cell visualized with

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brightfield (top), EGFP (green; middle), and Hoechst dye (blue; bottom). The presence of condensed chromatin in the presence of Btf$_s$ indicates cell death by apoptosis. The cells were photographed with an original

5   magnification of 1000X.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides cell lines and screening assays and reagents for identifying and

10   characterizing compounds which regulate apoptosis.

The tumor suppressor p53 gene product is a negative regulator of cellular growth and transformation (Ko and Prives 1996; Levine 1997; Vogelstein and Kinzler 1992; White 1996). Many studies have demonstrated that p53

15   functions as a transcriptional regulator by sequence-specific DNA binding (El-Deiry et al. 1992; Funk et al. 1992; Pietenpol et al. 1994). The p53 gene product regulates transcription by activation or repression (Ko and Prives 1996). DNA damage increases p53 levels which

20   promotes cell cycle arrest allowing DNA repair to occur. Alternatively DNA damage can induce apoptosis presumably when such damage is beyond repair (Ko and Prives 1996; Levine 1997; White 1996). The growth arrest function of p53 is predominantly implemented by transactivation of

25   the cyclin dependent kinase inhibitor *p21/WAF1/CIP1* (El-Deiry et al. 1993). p53 can also induce apoptosis by up-regulating the death-promoting *bax* gene (Miyashita and Reed 1995). Other transcriptional targets for p53 include *GADD45* (Kastan et al. 1992), *mdm2* (Barak et al.

30   1993; Wu et al. 1993), *cyclin G* (Okamoto and Beach 1994), and *IGF-BP3* (Buckbinder et al. 1995). The DNA binding ability of p53 plays a major role in regulating cell growth. The most frequently occurring p53

mutations in human tumors are found in this DNA binding domain (Hollstein et al. 1991; Ko and Prives 1996). Accordingly, intact p53 transcriptional functions are important to maintain genomic integrity.

5   The *mdm2* gene was originally cloned because of its amplification in a spontaneously transformed murine BALB/c cell line (Fakharzadeh et al. 1991). The human homologue of Mdm2 protein is a negative regulator of p53. Mdm2 protein inhibits p53-mediated functions of G_1

10   arrest and apoptosis (Chen et al. 1996), most likely by binding to the N-terminal transactivation domain of p53 (Momand et al. 1992; Oliner et al. 1993). Furthermore, Mdm2 appears to direct p53 degradation via the ubiquitin pathway (Haupt et al. 1997; Kubbutat et al. 1997). The

15   *mdm2* promoter contains p53 binding consensus sequences where p53 binds and positively regulates its expression, creating a negative feedback loop for regulating the activity and levels of p53 (Barak et al. 1993; Haupt et al. 1996; Wu et al. 1993). The functional

20   interdependence of Mdm2 and p53 was exemplified in studies with knock-out mice. Loss of Mdm2 resulted in early embryonic lethality which was rescued by deletion of p53 (Donehower et al. 1992; Montes de Oca Luna et al. 1995). Thus, Mdm2 is required *in vivo* for down-

25   modulation of p53 function and perturbation of this regulation can be deleterious to embryonic development.

The CBP/p300 family members regulate transcription by functioning as transcriptional coactivators. Although the precise mechanism of how transcriptional

30   adaptors function is not known, CBP/p300 and an interacting protein, P/CAF, have been shown to have histone acetyltransferase activity (Bannister and Kouzarides 1996; Ogryzko et al. 1996; Yang et al. 1996),

implicating a role for histone acetylation in
transcriptional regulation. These proteins also
interact with several transcription factors such as the
TAFs (Thut et al. 1995), TBP (Abraham et al. 1993), CREB

5   (Chrivia et al. 1993; Kwok et al. 1994), c-Jun/v-Jun
(Bannister and Kouzarides 1995), c-Myb/v-Myb (Dai et al.
1996), c-Fos (Bannister and Kouzarides 1995) and others,
which may determine the specificity of the regulation.
The p300 family of proteins has been recently shown to

10   bind to p53 and function as coactivators of p53-
inducible genes (Avantaggiati et al. 1997; Gu et al.
1997; Lill et al. 1997; Scolnick et al. 1997). The N-
terminal activation domain of p53 directly interacts
with the carboxy-terminal of p300 (Gu and Roeder 1997).

15   It has also been shown that p300 can acetylate the C-
terminal domain of p53 and that this modification
increases the sequence-specific DNA binding ability of
p53 (Gu and Roeder 1997). Thus, acetylation of specific
transcription factors may reflect one level of p300

20   transcriptional regulation.

The adenoviral early region 1 (E1) genes encode for
proteins that aid in cellular transformation by
activating proliferation and suppressing apoptosis
(White 1993; White and Gooding 1994). Expression of the

25   adenoviral E1A gene stimulates cell cycle progression by
interacting with and subverting the function of cellular
proteins required for normal cell cycle and
transcription regulation. E1A interacts with the
retinobalstoma (Rb) gene product as well as it's family

30   members, p107 and p130 (Dyson and Harlow 1992; Moran
1993; Whyte et al. 1988). E1A also binds to and
sequesters p300 (Eckner et al. 1994; Moran 1993; Yang et
al. 1996). E1A interactions with these cellular

proteins are important for transformation as suggested
by the observation that E1A mutants that fail to
interact with these proteins are incapable of promoting
transformation. Expression of E1A alone, however, is
insufficient to transform primary baby rat kidney (BRK)
cells because cell cycle deregulation by E1A also
stimulates p53-dependent apoptosis. Binding of p300 to
E1A cosegregates with induction of p53 and stabilization
(Chiou and White 1997; Querido et al. 1997; Sanchez-
Prieto et al. 1995). Transcriptional activation of p53
target genes, such as *bax* (Han et al. 1996; Sabbatini et
al. 1995), followed by induction of caspase activation
implements cell death (Rao and White 1997; Sabbatini et
al. 1997). Transcriptional activation of the p53 target
gene *p21^{WAF1}* is also induced, which contributes to
implementation of cell cycle arrest by p53, which is not
apparent due to cell death (Han et al. 1996; Sabbatini
et al. 1995).

     E1A-induced cellular transformation is sustained by
coexpression of the Bcl-2 adenoviral homologue, E1B 19K
or Bcl-2 itself, which inhibit E1A-induced, p53-mediated
apoptosis. E1B 19K and Bcl-2 inhibit apoptosis in part
by binding to the death promoting Bax protein (Han et
al. 1996). Thus E1B 19K or Bcl-2 expression inhibits
p53-mediated apoptosis but not growth arrest (Chiou et
al. 1994; Debbas and White 1993; Han et al. 1996;
Sabbatini et al. 1995). These cooperative functions
between early adenoviral genes stimulating cell cycle
progression and inhibiting apoptosis ensures efficient
transformation.

     In accordance with the present invention it has
been discovered that p300 is specifically required for
transactivation of the *mdm2* gene by p53 and also for

regulating p53-mediated apoptosis. Cells expressing E1A
were unable to up-regulate Mdm2, causing p53 to be
induced to high levels which resulted in p53-dependent
apoptosis. In contrast, BRK cells expressing c-Myc

5   instead of E1A, upregulated Mdm2, causing p53 down-
regulation and inhibition of p53-dependent apoptosis.
Thus, the inability to down-regulate p53 is associated
with apoptosis and provides an explanation for the
differences between the p53-dependent apoptotic response

10   of E1A versus Myc transformed cells.

       The adenovirus E1B 19K protein is a potent
inhibitor of apoptosis and cooperates with E1A to
transform primary rodent cells. E1B 19K shows sequence
and functional homology to the mammalian anti-apoptotic

15   gene product, Bcl-2. Like Bcl-2, the biochemical
mechanism of E1B 19K function includes binding to and
antagonizing cellular pro-apoptotic proteins, such as
Bax, Bak, and Nbk/Bik. In addition, there is evidence
that E1B 19K can affect gene expression, but whether

20   this contributes to the anti-apoptotic function of 19K
has not been determined. In an effort to further
understand the functions of E1B 19K, we screened for
19K-associated proteins using the yeast two-hybrid
system. A novel protein Btf (Bcl-2-associated

25   transcription factor) was identified that interacts with
E1B 19K, as well as with anti-apoptotic family members,
Bcl-2 and Bcl-xL, but not the pro-apoptotic protein Bax.
btf is a widely expressed gene that encodes a protein
with homology to the basic zipper (bZip)- and Myb-DNA

30   binding domains. Btf binds DNA in vitro and represses
transcription in reporter assays. E1B 19K, Bcl-2, and
Bcl-xL sequester Btf in the cytoplasm and block its
transcriptional repression activity. Expression of Btf

also inhibited transformation by E1A with either E1B 19K
or mutant p53, suggesting a role in either promoting
apoptosis or cell cycle arrest. Indeed, the sustained
over-expression of Btf in HeLa cells induced apoptosis
which was inhibited by E1B 19K. Furthermore, the
chromosomal localization of btf (6q22-23) maps to a
region that is deleted in some cancers, consistent with
a role for Btf in tumor suppression. Thus, btf may
represent a novel tumor suppressor gene, residing in a
unique pathway by which the Bcl-2 family can regulate
apoptosis.

These observations have facilitated the design of
recombinant cell lines that may be used to advantage to
screen therapeutic agents that regulate apoptosis.

The nucleic acids of the invention are sometimes
referred to herein as "isolated nucleic acids". This
term, when applied to DNA, is intended to signify a DNA
molecule that is separated from sequences with which it
is immediately contiguous (in the 5' to 3' directions)
in the naturally occurring genome of the organism from
which it was derived. For example, the "isolated
nucleic acids" of the invention may comprise a DNA
molecule inserted into a vector, such as a plasmid or a
virus vector, or integrated into the genomic DNA of a
procaryote or eucaryote. With respect to RNA molecules
of the invention, the term "isolated nucleic acids"
primarily refers to RNA molecules encoded by isolated
DNA molecules as defined above, or produced by synthetic
methods.

The term "transcription control (or regulatory)
element" as used herein, refers to an isolated DNA
segment that, under specified conditions, possesses a
transcription-regulating activity with respect to the

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expression of a transcription unit (i.e., a gene, as defined below).

The term "enhancer" is used herein to refer to a type of transcription control element. Enhancers generally activate the expression of a cis-linked gene.

The terms "transcription control element," "transcription regulatory element" and "enhancer" are used interchangeably herein when referring to the isolated nucleic acid molecules of the present invention.

The term "transcription factor" refers to protein factors that interact with DNA to enhance or repress transcription.

The term "repressor" refers to a transcription factor that inhibits gene expression upon interaction with DNA.

The term "transcription unit" refers to a nucleic acid molecule comprising one or more sequences which encode a gene product (usually a protein) and which is operably linked to a promoter or other regulatory sequences (enhancers etc.) necessary for expression of the coding sequence. The term "gene" is often used interchangeably with the term "transcription unit".

The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of transcription units and other transcription control elements (e.g. enhancers) in an expression vector.

The term "reporter gene" refers to a gene whose expression may be assayed; such genes include, without

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limitation, *lacZ*, amino acid biosynthetic genes, e.g.,
the yeast *LEU2, HIS3, LYS2,* or *URA3* genes, nucleic acid
biosynthetic genes, the mammalian chloramphenicol
transacetylase (CAT) gene, the luciferase gene, the

5   green fluorescent protein (GFP) or any surface antigen
gene for which specific antibodies are available.

        A "promoter" is a DNA sequence located proximal to
the start of transcription at the 5' end of an operably
linked transcribed sequence. The promoter may contain

10   one or more regulatory elements or modules which
interact in modulating transcription of the operably
linked gene.

        The term "tumor suppressor", as used herein,
relates to any protein/polypeptide inhibiting growth of

15   tumor cells in-vitro and/or in-vivo. Growth inhibition
involves mechanisms such as control of apoptosis and/or
of cell cycle progression as well as mechanisms
unidentified so far. "Tumor suppressors" are proteins
displaying biological activities identical to or similar

20   to those of p53, Rb (retinoblastoma gene product), WT
(Wilms tumor suppressor gene), VHL (von Hippel-Lindau
tumor suppressor gene), BRCA1 (breast cancer
susceptibility gene) and p16 (cyclin-dependent kinase
inhibitor).

25

I.   METHODS FOR CONSTRUCTING PLASMIDS AND PRODUCING THE
       CELL LINES OF THE INVENTION

        The cell lines of the invention may be prepared
30   according to the general methods set forth below for
plasmid construction, transformation of cultured cells,
and maintenance of cell lines.

A.   Plasmids

The plasmids of the invention may be constructed using conventional cloning methods.   A variety of different plasmid vectors are commercially available. Protocols for constructing such plasmids are provided in Current Protocols in Molecular Biology, Ausubel et al. (editors), John Wiley & Sons, Inc. 1987-1997.   Detailed protocols for constructing the plasmids of the invention are provided hereinbelow.

B.   Cell Lines

To achieve stable gene transfer, plasmid DNA must first be introduced into host cells.   This may be accomplished according to numerous methods known in the art, including but not limited to 1) calcium phosphate precipitation; 2) electroporation; and 3) liposome-mediated transfection.   For general protocols, see, e.g., Chapter 9 in Current Protocols in Molecular Biology, Ausubel et al. (editors), John Wiley & Sons, Inc. 1987-1997.

In accordance with the present invention, cell lines can be generated by co-transfection of a plurality of plasmids.   Alternatively, a sequential transfection can be performed, whereby a first cell line is generated that has stably incorporated a plasmid containing a p300 regulated promoter, such as the mdm2 promoter, operably linked to a reporter gene such as CAT.   This cell line can then be transfected in turn with a plasmid encoding wild type p300 to augment endogenous p300 levels.

Stable transfectants are selected by the ability of an individual cell colony to grow in the presence of a selected antibiotic, by virtue of a resistance-encoding gene carried on the transfecting plasmid DNA and

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incorporated into the genome of the cell. In a
preferred embodiment of the invention, both of the
plasmids used would contain a selectable marker gene for
double selection of stable transfectants by antibiotic

5   resistance.

II.   USES OF CELL LINES FOR SCREENING AGENTS THAT
REGULATE   APOPTOSIS

10

     The recombinant cell lines of the invention may be
used to identify and characterize therapeutic compounds
that act as p300 inhibitors and thereby induce
apoptosis.

15   For example, cells may be transfected with a
plasmid containing a reporter gene operably linked to a
promoter which is transactivated by p300. An exemplary
promoter is the mdm2 promoter. The cells would also be
co-transfected with a control plasmid containing a

20   second reporter gene operably linked to a non-p-300
responsive promoter.   The cell line may optionally be
stably transfected with a cDNA encoding p300 to augment
endogenous p300 levels.   Such cell lines may be used in
reporter assays for compounds that inhibit p300-

25   dependent transcription. These cells lines can be
screened for repression of the mdm-2 linked reporter by
compounds that do not alter expression of the non-p300
responsive reporter gene.

     Another strategy entails assaying p300 directly for

30   molecules that bind to the E1A binding site on p300.
Since binding of E1A to p300 inhibits p300 function, any
molecule or compound that mimicked E1A, should be a
potential p300 inhibitor. An E1A mutant that fails to

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bind p300 may be used as a control in such an assay.

Additonally, matched cell lines derived from the LTR 1A (myc +tsp53[val135]) cell line, exemplified herein, can be used in an in vivo cell-based screen for p300 inhibitors which, like E1A, promote p53 stabilization by inhibiting mdm-2 transcription and cause apoptosis. LTR 1A cells have intact p300 function and the ts p53 at the permissive temperature retains the capacity to activate the endogenous mdm-2 gene which is required for cell survival. Potentially therapeutic compounds that inhibit p300 function would cause apoptosis at the permissive temperature. This apoptotic phenotype can be used to advantage to identify agents of interest. LTR 1A cells that ectopically express Mdm-2 from a heterologous promoter would not be susceptible to p300 inhibition and are used as a control for p300 specificity.

The availability of the DNA sequences encoding Btf and Bcl-2 also facilitates the design of additional cell lines and screening methods to identify agents which regulate apoptosis via this pathway. Btf and Bcl-2 may be overexpressed and agents that disrupt Btf/Bcl-2 binding assessed. Disruption of Btf/Bcl-2 binding may be assess using co-immunoprecipitation for example. Agents so identified are expected to induce apoptosis in the targeted cell. Detailed methods for detecting apoptosis in cells are provided hereinbelow.

Antibodies specific for Btf are may be used to assess tumor samples for the presence or absence of Btf protein. Methods for generating antibodies and antibody fragments are well known to those of ordinary skill in the art. Screening methods employing such antibodies

include but are not limited to Western blotting, immunofluorescence and affinity chromatography.

Oligonucleotide probes and primers may also be used to assess expression of Btf in tumor samples in the clinical setting. DNA would be isolated from the tumor sample in question and assessed for the presence or absence of btf encoding nucleic acids. In accordance with the present invention, nucleic acids having the appropriate level of sequence homology with Sequence I.D. No. 1 may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al., (supra) using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989):

$T_m = 81.5°C + 16.6Log [Na+] + 0.41(\% G+C) - 0.63 (\% formamide) - 600/\#bp$ in duplex

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is

57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

5   If a tumor cell is found to contain a Btf deletion, the tumor cells may be genetically engineered to express the btf gene. Methods for delivering nucleic acids to target cells include retroviral targeting vectors and adenoviral vectors.

10

The following materials and methods are provided to facilitate the practice of the present invention.

15   *Cells and culture conditions*

Primary Fisher BRK cells were prepared from 6 day old baby rats and cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum as described previously (White et al. 1991). Plasmids were

20   transfected by electroporation, and cells were maintained in DMEM with 10% fetal bovine serum. The transformed BRK cell line, p53A, was derived from transfection of pCMVE1A and pLTRcGval135 plasmids (Debbas and White 1993). The 19K1 and 4B cell lines

25   were derived from transfecting pCMV19K and pSVBcl-2, respectively, and a neomycin-resistance plasmid, pSVneo, by electroporation (Debbas and White 1993) (Chiou et al., 1994; Sabbatini et al., 1995). Control cell line, p53An1, was derived from p53A cells containing only the

30   neomycin-resistance plasmid (Debbas and White 1993). The LTR.1A cells were generated by transfecting primary BRK cells with 10 µg of LTR H-myc, which expresses human c-Myc, and pLTRcGval135 as described previously

(Sakamuro et al. 1995).

Antibodies

SMP14, a mouse monoclonal anti-Mdm2 antibody (Santa

5   Cruz Biotechnology, Inc, Santa Cruz, CA) was used at

1:1000 (Western) or 1:100 (immunofluorescence) to detect

Mdm2.   Clone 248, a murine-specific anti-p53 mouse

monoclonal antibody which was kindly provided by Dr. A.

Levine was used at 1:5000 to detect p53.   Mouse

10   monoclonal anti-p21^{WAF1} (Ab-4, Calbiochem, San Diego, CA)

was used at 1:50 dilution to detect p21^{WAF1}.   A rabbit

polyclonal anti-Bax (P-19, Santa Cruz) was used at 1:100

to detect Bax and a mouse monoclonal anti-actin antibody

(Amersham) was used at 1:1000 dilution to detect actin.

15   Anti-HA mouse monoclonal antibody (Babco, Richmond, CA)

at 1:1000 dilution was used to detect ectopically

expressed p300.   A mouse monoclonal anti-E1A antibody

M73 (Calbiochem) was used at 1:1000 dilution to detect

E1A.   A polyclonal anti-E1B 19K antibody was used at

20   1:10,000 to detect transiently expressed E1B 19K.

Cell viability and morphology

Cell viability was measured by trypan blue

staining.   Cells were trypsinized, centrifuged,

25   resuspended in PBS, and counted using a hemocytometer

after diluting in trypan blue.   Apoptotic morphology was

assessed by scoring for cells that are positive by

fluorescence microscopy and are rounded and non

adherent.   Cell morphology was also documented by

30   photography at 100 x magnification on a Nikon phase-

contrast microscope and camera using Kodak film.

Western Blot Analysis

Cells were incubated at 37.5°C and 32°C for various time intervals prior to lysing in buffer containing 4% SDS and 5% β-mercaptoethanol. Equal amounts of protein
5   (20-30 μg) were electrophoresed on 7.5-12% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked for 15 minutes at room temperature in 5% nonfat Carnation dry milk in PBS containing 0.1% Tween-20 (PBST). Membranes were incubated with primary antibody
10   followed by washes in PBST and then incubated with horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG monoclonal antibody at 1:2000 dilution. After several washes, blots were developed using the ECL chemiluminescence detection kit
15   according to manufacturer's recommendations (Amersham LIFE SCIENCES, Arlington Heights, IL).

Indirect immunofluorescence and microscopy

Cells were fixed in cold methanol prior to
20   incubation with primary antibodies. Subsequent to several washes in PBS, cells were incubated with rhodamine-conjugated goat anti-mouse antibody or fluorescein-conjugated goat anti-rabbit antibody. Cells were then washed, mounted and analyzed by fluorescence
25   microscopy using a Nikon FXA epifluorescence microscope.

Northern Blot Analysis

Cytoplasmic RNA was extracted from p53A and 19K1 cells using the NP-40 lysis protocol as previously
30   described (Muraoka et al. 1996). Northern blots were performed by loading 30 μg of cytoplasmic RNA on a formaldehyde gel and blotted as previously described (Muraoka et al. 1996) using Hybond-N membranes

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(Amersham) for transfer. The membrane was hybridized with a random-primed labeled murine *mdm2*-specific probe in ExpressHyb Hybridization solution (CloneTech, Palo Alto, CA) at 65°C. Blots were washed in 0.1X SSC and 0.1% SDS at 50°C and exposed to film for 2 days.

Transient Transfections

The indicated amount of plasmid DNA was transfected by electroporation. After transfection, cells were incubated at 37.5°C for 24 hours to recover, and were then shifted to 32°C for 24 hours before analysis for viability by trypan blue staining and apoptotic morphology. For p300 transfections, p53A cells were electroporated with 12 or 24 μg of pCMVβ alone, pCMVβp300 or pCMVp300Δ30 mutant (deletion in E1A-interacting domain) (Arany et al. 1994). For transient expression of E1A proteins, LTR.1A cells were electroporated with 10 μg of pCMVβ alone, pCMV12SE1A, pCMV12S.RG2, or pCMV12S.YH47.928 (Wang et al. 1992). For Mdm2 transient expression, p53A cells were electroporated with 5-10 μg of pCMVβ alone, pCMVβ-X$_2$ (wild-type Mdm2), and pCMVβ-ΔXM (mutant Mdm2 with deletion of the N-terminal p53-binding domain) (Haupt et al. 1997).

Stable transfections

Cells were tranfected by electroporation using 2 μg of pCMVβ alone or pCMVβ-X2 and 200 ng of pSVneo along with 10 μg of salmon sperm DNA. Cells were selected in complete media containing 0.5 mg/ml G418 (Sigma) for a week and then incubated at the permissive temperatute to select for cells stably expressing Mdm2. Two independent pools of p53A cells stably expressing wild-

type Mdm2 were analyzed.

Yeast two-hybrid system for identifying E1B 19K binding proteins

5   Procedures for utilizing the two-hybrid system to identify E1B 19K binding proteins were described previously (Han et al., 1996). A HeLa cDNA library was constructed in the pGAD-GH vector and screened against pGBT9-E1B 19K. Plasmids were transformed into *S.*
10   *cerevisae* YGH1 cells and positive clones were selected based on growth in the absence of histidine and production of β-galactosidase. False-positive clones were eliminated by testing for interactions with an irrelevant hydrophobic protein (Apc-2) and the empty
15   pGBT9 vector. Missense and deletion mutants of E1B 19K tested for interaction with BP-1 were described previously (Han et al., 1996, supra). pGBT8-Bcl-2 (20) and pGBT9-Bax (26) were also described previously. pGBT8-Bcl-x$_L$ was kindly provided by Dr. G. Nuñez (Univ.
20   of Michigan, Ann Arbor, MI).

        The full length transcript, *btf*$_s$ was identified by screening a λ-cDNA library prepared from HeLa cells (Stratagene, La Jolla, CA) using conventional techniques. The cDNA sequence of *bp-1* and the
25   subsequent full length *btf* were analyzed using Sequenase 2.0 (US Biochemical, Cleveland, OH) according to the manufacturer's specifications and later confirmed by fluorescent terminator cycle sequencing using an automated 377 DNA sequencer (Perkin-Elmer, Applied
30   Biosystems, Foster City, CA).

Plasmid construction

        A PCR product of *btf*$_s$ from the λ-screen was digested

with *XmaI* and *NotI* (blunt) and ligated into both pGAD-GH
cut with *XmaI-XhoI* (blunt) and pGBT9 cut with *XmaI-SalI*
(blunt). To prepare pcDNA3-Myc-Btf$_s$ for mammalian
expression and *in vitro* translation, oligonucleotides
5   encoding a Myc epitope with flanking *KpnI* and *XmaI* sites
were annealed with a PCR cloned fragment of *btf$_s$* from
bluescript digested with *XmaI* and *NotI*, and pcDNA3
(Invitrogen, San Diego, CA) digested with *KpnI* and *NotI*.
The resulting plasmid encodes a Myc epitope at the N-
10   terminal end of Btf$_s$. Btf$_s$ and two deletion mutants were
fused in frame with the GAL-4 DNA-binding domain in the
pm1 vector for use in transcriptional reporter assays
(kindly provided by Dr. C. Abate-Shen, Center for
Advanced Biotechnology and Medicine, Piscataway, NJ).
15   The full-length *btf$_s$* construct was prepared by digesting
pGBT9-Btf$_s$ with *XmaI* and *PstI* and ligating into the same
sites in the pm1 vector. The N552 and C210 mutants were
generated by digesting pGBT9-Btf$_s$ with *EcoRI-PstI* and
XmaI-BamHI, respectively, and ligating into the same
20   restriction sites within pm1. *btf$_s$* was also cloned into
pIRES-EGFP (Clontech, San Francisco, CA) for cell cycle
analysis of Btf$_s$ expressing cells. The construct was
prepared by digesting pcDNA3-Myc-Btf$_s$ with *SmaI* and *NotI*
and ligating to pIRES-EGFP cut with *EcoRV* and *NotI*. The
25   pcDNA3-Bcl-2 plasmid was prepared by ligating an *EcoRI-
XhoI* fragment from pSFFV-Bcl-2 (Hockenberry et al.,
1990) (provided by Dr. S. Korsmeyer, Washington
University School of Medicine, Saint Louis, MO) into
pcDNA3. The C-terminally V5/His tagged E1B 19K was
30   prepared by TA cloning into pcDNA3.1/V5/His-TOPO
(Invitrogen, San Diego, CA) according to manufacturer's
specifications.

Cell lines

The HeLa cells were maintained in culture in
Dulbecco modified Eagle medium (D-MEM) with 10% fetal
bovine serum (FBS) at $37°C$ / 5% CO_2. Stable HeLa cell
lines containing Bcl-x_L were prepared by electroporating
1 μg of pcDNA3-Flag-Bcl-x_L (Merino et al., 1995),
provided by Dr. G. Nuñez (Univ. of Michigan, Ann Arbor,
MI), into HeLa cells and selecting with 1.2 mg/ml
geneticin. Expression of Bcl-x_L was verified by
immunofluorescence.

Northern blot for btf expression

Northern blot analyses were performed using
commercially available blots of multiple tissues or
cancer lines (Clontech, San Francisco, CA). Each lane
contained 2 μg of the indicated poly A^+ RNA. The *btf_s*
probe was prepared by random priming using a purified
*Xma*I-*Xho*I fragment from pGAD-GH-Btf$_s$. Human β-*actin* cDNA
supplied with the blots were used as a control probe to
confirm equal mRNA loading. Hybridization was performed
using ExpressHyb solution (Clontech, San Fransisco, CA)
according to manufacturer's specifications. Bands were
visualized by autoradiography and using a PhosphorImager
(Molecular Dynamics, Sunnyvale, CA).

In vitro binding assay

Binding reactions were performed by combining [35][S]-
methionine-labeled *in vitro* translated Myc-Btf$_s$ or Myc-
Bax (Han et al., 1996) with V5/His-E1B 19K, Bcl-2, Flag-
Bcl-x_L, or luciferase (Promega Corp., Madison, WI)
prepared using the TnT T7 reticolocyte lysate system
(Progema Corp., Madison, WI) according to manufacturer's
specifications. Samples were incubated with anti-Myc

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monoclonal antibody (Oncogene Research Products,
Cambridge, MA) in 500 μl of NETN buffer [20 mM Tris (pH
8.0), 100 mM NaCl, 1 mM EDTA, and 0.2% NP-40] for 1.5
hrs at 4°C followed by protein A-sepharose. All samples

5   were then washed three times in NETN buffer, resuspended
in 2X Laemmli buffer, boiled for 5 minutes, and analyzed
by SDS-PAGE. Gels were fixed in 50% methanol, 10%
acetic acid for 1 hour, dried and then visualized by
autoradiography.

10

DNA binding assay

^{35}[S]-Methionine labeled *in vitro* translated Myc-
Btf$_s$ and E1B 19K were prepared from 1 μg of pcDNA3-Myc-
Btf$_s$ and pcDNA3-E1B 19K (Han et al., 1996, supra) using

15   the TnT T7 reticolocyte lysate system. The proteins
were incubated with 200 μl of native DNA-cellulose
(Pharmacia Biotech, Piscataway, NJ) in 500 μl of NETN
buffer for 2 hrs at 4°C. The relative levels of Btf$_s$ and
E1B 19K production were determined by

20   immunoprecipitation with anti-Myc (Oncogene Research
Products, Cambridge, MA) or anti-E1B 19K (White and
Cipriani, 1989) in NETN buffer followed by protein A-
sepharose and then washed in NETN. Samples were
resolved by SDS-PAGE and examined by autoradiography as

25   described above.

Indirect immunofluorescence

HeLa cells were electroporated with 15 μg of
pcDNA3-Myc-Btf$_s$ along with 15 μg of either pCMV-E1B 19K

30   (White and Cipriani, 1989, supra) or pcDNA3-Bcl-2.
Total amount of DNA was kept constant using empty pcDNA3
vector. Cells grown on glass coverslips were stained 24
hours post-transfection as described previously (Perez

33

and White, 1998). Briefly, the cells were fixed with
2% paraformaldehyde in Phosphate Buffered Saline (PBS),
and then permeabilized with 0.5% Triton X-100 in PBS.
The cells were double labeled with an anti-Myc mouse
5   monoclonal antibody (Oncogene Research Products,
Cambridge, MA) along with either an anti-E1B 19K rabbit
polyclonal (White and Cipriani, 1989, supra) or an anti-
Bcl-2 hamster monoclonal antibody (PharMingen, San
Diego, CA). Antibody complexes were visualized with
10   rhodamine-conjugated goat anti-mouse antibody along with
the fluorescein-conjugated antibodies goat anti-rabbit
or goat anti-hamster (Jackson ImmunoResearch
Laboratories, Inc., West Grove, PA).
Immunocytochemistry was also performed in HeLa-Bcl-x$_L$
15   cells electroporated with 15 μg of pcDNA3-Myc-Btf$_s$.
These cells were stained for the Myc epitope as
described above. Expression of Flag-tagged Bcl-x$_L$ was
confirmed by staining separate coverslips with anti-Flag
M5 monoclonal antibody (Scientific Imaging Systems, New
20   Haven, CT) and rhodamine-conjugated goat anti-mouse.
The cells were visualized by epifluorescence with a
Nikon FXA microscope (Nikon Inc., Garden City, NY).

Transcription assays

25   Transcriptional reporter assays were performed as
described previously (Catron, et al., 1995). HeLa cells
were plated onto 60 mm tissue culture dishes and grown
to 50-75% confluency. The cells were then transfected
with 2.5 μg of a GAL-4 luciferase reporter construct
30   (kindly provided by Dr. C. Abate-Shen, Center for
Advanced Biotechnology and Medicine, Piscataway, NJ)
along with 2.5 μg of both a pm1 vector (Btf$_s$, Btf$_s$-N552
or Btf$_s$-C210) and a *bcl*-2 family gene (pCMV-E1B 19K,

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pcDNA3-Bcl-2, or pcDNA3-Flag-Bcl-x$_L$). DNA concentrations
were kept constant using appropriate empty vectors,
either pml or pcDNA3. The cells were transfected using
SuperFect (Qiagen, Valencia, CA) according to the

5 manufacturer's specifications and harvested 24 hours
post-transfection. Expression of mutant and wild-type
Btf$_s$ proteins were verified by immunofluorescence using
an antibody against the GAL-4 DNA-binding domain
(Clontech, San Fransisco, CA). Luciferase activity was

10 determined using the Luciferase Assay System (Promega
Corp., Madison, WI) measured in a scintillation counter,
and then normalized for protein concentrations measured
by a Bradford assay. Values were graphed as a percent
of the negative control (empty pml, empty pcDNA3, and

15 GAL-4 luciferase).

Transformation assay

Transformation assays of baby rat kidney (BRK)
cells were performed as described previously (White et
al., 1992). Briefly, BRK cells prepared from 6-day-old

20 Fisher rats were electroporated with carrier DNA along
with linerized test DNA (15 μg pCMV-E1A (White et al.,
1991), 15 μg pCMV-E1B 19K or pCMV-p53DD (Shaulian, et
al., 1992), and 45 μg pcDNA3-Myc-Btf$_s$). DNA

25 concentrations were kept constant using appropriate
empty vectors. The cells were cultured for 3 to 4 weeks
at 38.5°C in D-MEM supplemented with 5% FBS, and then
stained with Giemsa. Foci were counted from four dishes
per condition.

30

Cell cycle analysis

HeLa cells were electroporated with 20 μg pIRES-
EGFP- Btf$_s$ or empty pIRES-EGFP vector in combinations

with 10 μg pCMV-E1B 19K or empty pcDNA3 vector. Cells were harvested 48 and 72 hours post-transfection and fixed in 2% paraformaldehyde in PBS for 30 min at 4°C. The cells were washed and then stained with PBS

5   containing 1 μg/ml propidium iodide, 250 μg/ml RNase A, and 0.1% Tween 20 for at least 30 min at room temperature. The fluorescent intensities for EGFP and propidium iodide were analyzed by FACS (EPICS PROFILE-II; Coulter, Miami, FL). In addition, the live

10   transfected cells were stained with Hoechst dye to visualize the DNA and photographed with a Nikon FXA microscope (Nikon Inc., Garden City, NY).

15   The following examples are provided to illustrate particular embodiments of the invention. They are not intended to limit the invention in any way.

EXAMPLE I

20   *Mdm2 expression regulates p53-dependent apoptosis in BRK cells*

BRK cells were transformed by a temperature sensitive p53 (val135) mutant and either adenovirus E1A (p53A) or c-Myc (LTR.1A). As previously reported, both

25   types of cell lines expressed the temperature sensitive p53 (val135) mutant in which p53 is in the wild-type conformation at the permissive temperature of 32°C and in the mutant conformation at the nonpermissive temperature of 37.5°C (Debbas and White 1993; Sakamuro

30   et al. 1995). There are two striking differences between E1A + tsp53(val135) transformed (p53A) and myc + tsp53(val135) transformed (LTR.1A) cell lines. First, BRK cells expressing E1A undergo massive apoptosis at

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the permissive temperature (Chiou et al. 1994; Debbas
and White 1993; Sabbatini et al. 1995) whereas the Myc
expressing cells undergo a wave of apoptosis within the
first 24 hours at the permissive temperature but are
5   predominantly resistant to apoptosis (Sakamuro et al.
1995).   Second, E1A expressing cells maintain high
levels of p53, whereas the p53 levels are down-regulated
at 32°C in Myc expressing cells (Debbas and White 1993;
Sakamuro et al. 1995).   Thus, high levels of p53
10   correlate with augmentation of the apoptotic response in
E1A expressing p53A cells.   Since E1A stabilizes p53
(Chiou et al. 1994; Lowe and Ruley 1993) and this
activity requires that E1A binds to p300 (Chiou and
White 1997), this suggests a possible function for
15   sequestration of p300 and induction of p53 levels.
Furthermore, recent reports demonstrate the requirement
for p300 in p53 transactivation (Gu et al. 1997; Lill et
al. 1997; Scolnick et al. 1997), and a role for Mdm2 in
promoting p53 degradation (Haupt et al. 1997; Kubbutat
20   et al. 1997).   The possible role of p300 in the Mdm2-
dependent negative-feedback loop and the control of p53-
dependent apoptosis was further investigated.

In accordance with the present invention it was
determined whether the levels of p53-inducible gene
25   products Mdm2, p21^{WAF1} and Bax were affected by the p300-
binding protein E1A.   Western blot analysis shows that
p53 levels were up-regulated in E1A transformed cells
and down-regulated in Myc transformed cells at 32°C
(Fig. 1A) as previously reported (Debbas and White 1993;
30   Sakamuro et al. 1995).   A lower band which may represent
a proteolytic cleavage product of p53 was also observed
in p53A cells as apoptosis progressed at the permissive
temperature.   See Figure 1A.   High p53 levels correlated

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with apoptosis in p53A cells. See Figure 3A and Figure

4, top panel. Endogenous Mdm2 protein was initially

present but was down-regulated at 32°C in p53A cells as

shown by Western blot analysis (Figure 1) and

5   immunofluorescence (Figure 2, upper panel). Western

blot analysis of Mdm2 in p53A cells at the permissive

temperature showed a lower band around 50 kDa recognized

specifically by the anti-Mdm2 monoclonal antibody (data

not shown). This lower band may represent a cleavage

10   product of Mdm2. However, this band dissappeared as

cells were incubated at the permissive temperature.

Mdm2 down-regulation was surprising since *mdm2* is a p53-

regulated gene and suggested that p300 may be necessary

for Mdm2 transactivation by p53.

15   In c-Myc transformed LTR.1A cells, Mdm2 was up-

regulated and p53 levels were low at 32°C. See Figure

1B and Figure 2, lower panel). Therefore Myc, but not

E1A expressing BRK cells demonstrate p53-dependent

induction of *mdm2*. Furthermore, high levels of Mdm2 in

20   Myc transformed BRK cells may have promoted p53

degradation as shown in Figure 1A. The LTR.1A cells

initially undergo apoptosis but rapidly become resistant

to p53-mediated cell death at 32°C (Figure 3, bottom

panel and Figure 4, bottom panel) which correlated with

25   the down-regulation of p53 and up-regulation of Mdm2.

Mdm2 is only one of many p53-inducible genes that

collectively act to modulate the physiological response

to p53 induction. Therefore, p21^{WAF1} and Bax levels were

also examined for transactivation in p53A and LTR.1A

30   cells. In p53A cells, Bax and p21^{WAF1} levels were induced

at 32°C even in the presence of E1A and inhibition of

p300 as shown in Figure 1A and Figure 1B (Han et al.

1996; Sabbatini et al. 1995). LTR.1A cells down-

regulated Bax but up-regulated low levels of p21^{WAF1} at 32°C. See Figure 1. Similar results (data not shown) were obtained with independent E1A + tsp53(val135) and c-Myc + tsp53(val135) transformed clones (Debbas and White 1993; Sakamuro et al. 1995). These results indicate that E1A and Myc differentially alter p53-dependent gene expression.

In order to determine whether the absence of Mdm2 protein in p53A cells reflected a transcriptional event, Northern blot analysis was performed using cytoplasmic RNA extracted from E1A transformed p53A cells incubated at the permissive temperature for the indicated time intervals. See Figure 5. Two transcripts of 3.3 and 1.7 kb hybridized to a *mdm2*-specific probe. Both transcripts, particularly the 3.3 kb, were down-regulated to some extent as cells were incubated at 32°C.   These results suggest that p300 may be required for Mdm2 transactivation, but may be dispensable for Bax and p21^{WAF1} regulation.

p300 is required for the up-regulation of Mdm2 by p53 and inhibition of p53-mediated apoptosis

We next attempted to rescue p53A cells from apoptosis at the permissive temperature by ectopically expressing p300. Vector alone, wild-type p300 and a functional but truncated form of p300 with a deletion of E1A binding domain (Arany et al. 1994), were transiently transfected in p53A cells. p53-dependent apoptosis was examined at the permissive temperature 48 hours post-transfection. p53A cells transiently expressed the exogenous wild-type p300 and the E1A binding mutant form of p300, neither of which were detected in mock transfected cells. See Figures 6 and 7. The up-

regulation of Mdm2 in p300 transfected cells was not detected by Western blot analysis above endogenous levels (Fig. 6) because the transfection efficiency was low in transient assays. However, the up-regulation of Mdm2 in both wild-type and E1A-binding mutant of p300 was easily observed in 90% of transfected cells by indirect immunofluorescence when individual transfected cells were examined as shown in Figure 7, lower panel, arrows. Furthermore, ectopic expression of wild-type and mutant p300 rescued cells from apoptosis as cell viability was elevated as much as 20-40% above mock transfected cells. See Figure 8. The mutant form of p300 which was unable to bind E1A, protected cells better than wild-type p300, presumably because it evades E1A sequestration.

In addition, we determined the percentage of cells with apoptotic morphology that were ectopically expressing wild-type p300 ectopically or the E1A-binding mutant of p300 following transient transfection with 24 μg of plasmid DNA. Apoptotic morphology was determined by counting cells that were rounded in shape and detaching from the dish. The percentage of cells with apoptotic morphology transiently expressing a control protein, a fragment of the lamin A protein (Rao et al. 1996), compared to p300 and mutant p300 transfected p53A cells at the permissive temperature was determined by indirect immunofluorescence. See Figure 9. Only 4-8% of the cells expressing wild-type p300 and mutant p300, respectively, have apoptotic morphology compared to 62% cells expressing the lamin fragment (Fig. 9). These results reaffirm that expression of wild-type p300 or the E1A-binding p300 mutant is sufficient to rescue cells from p53-mediated apoptosis.

In order to determine whether LTR.1A cells can be
made susceptible to p53-mediated death, wild-type E1A
(12S E1A) was tranfected into these cells. Transient
expression of 12S E1A in LTR.1A cells, see Figure 10,
5   promoted apoptosis at the permissive temperature as
shown in Figure 11. In addition, Mdm2 levels were down-
regulated in cells transfected with 12S E1A as detected
by Western blot analysis (Fig. 10).   Since E1A can also
bind to Rb, we transiently transfected LTR.1A cells with
10   12S E1A mutant (12S.RG2) that is unable to bind to p300
but retains it ability to bind to Rb, and a 12S E1A
mutant (12S.YH47.928) that binds to p300 but not to Rb
(Wang et al. 1992), and examined p53-dependent apoptosis
at the permissive temperature.   Cells transfected with
15   12S.RG2 did not down-regulate Mdm2 (Fig. 10) and were
resistant to p53-dependent apoptosis (Fig. 11).   In
contrast, cells transfected with 12S.YH47.928 down-
regulated Mdm2 (Fig. 10) and were susceptible to p53-
dependent apoptosis at the permissive temperature (Fig.
20   11).   These results suggest that the ability of E1A to
bind to p300 and inhibit Mdm2 induction correlates with
E1A-mediated, p53-dependent apoptosis.

Overexpression of Mdm2 inhibits p53-mediated apoptosis
25   To determine whether Mdm2 expression can bypass the
requirement for p300 cotransactivation and rescue p53A
cells from p53-mediated death, p53A cells were
transiently transfected with expression plasmids
encoding wild-type Mdm2 and a truncated form of Mdm2
30   lacking the N-terminal p53-binding domain (Haupt et al.
1997).   Transient overexpression of wild-type and mutant
Mdm2 were detected by Western blot analysis in p53A
cells (Fig. 12).   Transient transfection of cells with 5

or 10 μg wild-type Mdm2 rescued cells from p53-dependent
apoptosis at the permissive temperature (Fig. 13). The
truncated form of Mdm2 rescued cells from apoptosis less
efficiently than the wild-type protein (Fig. 13),

5   presumably because it was not able to bind to p53 and
promote its degradation. Furthermore, we examined the
cell morphology of p53A cells expressing a wild-type and
mutant Mdm2 plasmid DNA as compared to the morphology of
cells expressing a control lamin fragment incubated at

10   the permissive temperature using indirect
immunofluorescence (Fig. 14). The percentage of
apoptotic cells expressing wild-type and mutant Mdm2 was
only 10% and 30%, respectively, as compared to 56%
apoptosis in cells expressing the control lamin fragment

15   (Fig. 14). The Mdm2 mutant, however, possessed greater
apoptotic suppressing activity than the control lamin
fragment which may indicate that Mdm2 can suppress
apoptosis by a mechanism independent of p53 binding.
These results demonstrate that overexpression of Mdm2

20   can rescue E1A-expressing cells from p53-mediated
apoptosis by either promoting p53 degradation or by
inhibiting its activity by direct interaction.

E1B 19K or Bcl-2 can bypass E1A inhibition of p300 and
25   *restore mdm2 transactivation.*

        Another mechanism for suppressing p53-dependent
apoptosis is through expression of Bcl-2 or E1B 19K,
which function in part by binding to Bax and
inactivating its pro-apoptotic function. To test if Bcl-

30   2 or E1B 19K could also function to suppress apoptosis
by influencing p53 target gene expression, we examined
p53-inducible gene products, Mdm2, p21^{WAF1} and Bax, in
p53A cells stably expressing adenovirus E1B 19K (19K1)

or its human homolog Bcl-2(4B).

As reported previously, the expression of 19K or
Bcl-2 abrogates p53 dependent apoptosis at 32°C (Chiou
et al., 1994a; Sabbatini et al., 1995a; Han et al.,
5   1996). Western blot analysis indicated that p21^{WAF1} and
Bax levels increased at 32°C regardless of whether or
not 19K or Bcl-2 were expressed and the levels of p53
remained high. See Figures 15A , 15B and 15C. However,
the presence of 19K or Bcl-2 dramatically up-regulated
10   Mdm2 levels, whereas the control E1A + tsp53 (val135-
transformed p53A cells transfected with vector alone,
(p53An1) down regulated Mdm2 as expected (Fig. 15). This
suggested that E1B 19K or Bcl-2 could restore p53-
dependent mdm2 expression in p53A cells.
15   To determine whether the expression of E1B 19K or
Bcl-2 was sufficient for up-regulation of Mdm2 levels,
we transiently transfected 19K or Bcl-2 in p53An1 cells
and examined Mdm2 levels at 32°C. Western blot analysis
(Fig. 16), and indirect immunofluorescence indicated
20   that Mdm2 leves were up-regulated when either 19K or
Bcl-2 was expressed transiently.

To determine whether expression of E1B 19K
upregulates Mdm2 at the transcriptional level, we
performed Northern blot analysis using cytoplasmic RNA
25   from cells expressing E1B 19K (19K1) incubated at 32°C
for the indicated time intervals (Fig. 17) As shown in
Figure 17, mdm2 mRNA expression was up-regulated after
48 hour incubation at the permissive temperature. This
suggested that E1B 19K and Bcl-2 may restore mdm2
30   transactivation by p53, which would otherwise be
inhibited by E1A binding to p300. In addition, previous
studies have shown that Bax, and p21^{WAF1} mRNA and protein
were also up-regulated in 19K1 cells incubated at the

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permissive temperature. Therefore, the presence of 19K did not affect the expression of Bax or $p21^{WAF1}$, however, E1B 19K can directly bind to Bax and inhibit its function. E1B 19K and Bcl-2 have been shown to relieve

5 transcriptional repression by E1A and p53, which may be responsible for inhibition of apoptosis and Mdm2 may be a physiological target of this activity.

Discussion

10   E1A + tsp53(val135) transformed p53A cells were unable to transactivate *mdm2* as a result of E1A inhibition of p300, indicating that p53 requires p300 to transactivate *mdm2*. See Figure 18. Furthermore, low Mdm2 levels in p53A cells relieve the negative-feedback

15 regulation of p53 resulting in high p53 accumulation leading to apoptosis. In contrast, c-Myc + tsp53(val135) transformed LTR.1A cells transactivate Mdm2 in the absence of E1A inhibition of p300 (Fig. 18). Up-regulation of *mdm2* inhibits p53 function and promotes

20 p53 degradation (Haupt et al. 1997; Kubbutat et al. 1997), which in turn inhibits p53-mediated cell death resulting in growth arrest in LTR.1A cells. Cellular levels of p53 control the response of the cells such that lower levels of p53 induce growth arrest and higher

25 levels induce apoptosis (Chen et al. 1996). Therefore, it may be concluded that p300 regulation of Mdm2 levels determines whether the physiological response to p53 is growth arrest or apoptosis.

Interestingly, p300 cotransactivation is not

30 required for the other p53-inducible genes *Bax* and *p21^{WAF1}*. It has previously been reported that E1A inhibits p53-mediated transactivation using promoter reporter assays (Steegenga et al. 1996). Our studies,

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however, demonstrate that E1A specifically inhibits
endogenous *mdm2* transactivation and not other p53-
inducible genes such as *bax* and *p21^{WAF1}*. p300/CBP
functions by interacting not only with p53, but also

5   with other factors such as the TAFs (Thut et al. 1995),
TBP (Abraham et al. 1993), CREB (Chrivia et al. 1993;
Kwok et al. 1994), c-Jun/v-Jun (Bannister and Kouzarides
1995), c-Myb/v-Myb (Dai et al. 1996), c-Fos (Bannister
and Kouzarides 1995) and others. p53-binding alone may

10   not regulate the specificity of p300 cotransactivation,
however, as numerous other cellular interactions may
determine the specificity transactivation, which may
explain differential regulation of p53-inducible genes
by p300.

15   p300/CBP transcriptional coactivators have been
shown to have histone acetyltransferase activity which
can modify chromatin structure and enhance gene
expression (Bannister and Kouzarides 1996; Ogryzko et
al. 1996). Recently, p300 has been shown to acetylate

20   p53 itself (Gu and Roeder 1997). The acetylation of p53
increases the binding activity of p53 to specific
consensus sequences. *In vivo*, this activity may be
required for facilitating p53 tetramer interactions with
the DNA template, thereby promoting *mdm2*

25   transactivation.
           Mutations that inactivate p300 have been described
in colorectal and gastric carcinomas (Muraoka et al.
1996), which suggests that p300 functions as a negative
regulator of cell growth. Missense mutations of p300

30   coupled with the deletion of the second allele of the
gene were observed in these carcinomas. These
observations suggest that inactivation of p300 may play
a role in the development of cancer. E1A inhibition of

p300 transactivation may be one mechanism whereby this viral protein promotes cellular transformation. Moreover, p300 inactivation may render cells susceptible to agents that induce p53-mediated cell death such as UV

5   and ionizing radiation. It is intriguing to speculate that acetyltransferase inhibitors specific for p300 may be used in cells that do not respond to chemotherapy regardless of having wild-type p53.

10   **EXAMPLE II**

       As discussed previously, apoptosis is a genetically controlled process of cell suicide that plays a critical role in maintaining homeostasis and preventing disease. Disruption of apoptosis leads to impaired development,

15   cancer, neurodegenerative and autoimmune diseases, and sustained viral infection. The regulation of apoptosis is a precarious balance between factors that promote survival and those responsible for initiating and executing cell death. One major advance toward the

20   understanding of apoptosis regulation has been the characterization of the Bcl-2 family (White, E., 1996). This family consists of highly conserved proteins with opposing biological function. Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-x_L, inhibit

25   apoptosis triggered by many circumstances, including TNF-α, Fas, UV radiation, chemotherapeutic drugs, and growth factor/hormone withdrawal. In contrast, pro-apoptotic Bcl-2 family members, such as Bax, Bak, and Nbk/Bik, induce cell death in numerous model systems.

30   The adenovirus E1B 19K protein cooperates with E1A in transformation assays and is a viral homologue of mammalian Bcl-2. Expression of E1B 19K, or Bcl-2, inhibits E1A-induced, p53-mediated apoptosis (Chiou et

al, 1994; Han et al., 1996). Like Bcl-2, E1B 19K
interacts with and antagonizes several pro-apoptotic
family members, including Bax (Han et al., 1996),
Nbk/Bik (Boyd, et al., 1995), and Bak. However, it is
5   still unclear how these proteins affect cell survival or
death and whether binding to proteins unrelated to the
Bcl-2 family contribute to apoptosis or other cellular
processes.

While there has been some debate concerning the
10   biochemical function of E1B 19K and other Bcl-2 family
members in the regulation of apoptosis, recent studies
have revealed several possible mechanisms. Clearly,
interactions between Bcl-2 family members play an
integral part in the regulation of apoptosis and
15   transformation. A common feature of the Bcl-2 family is
the occurrence of protein-protein interactions between
the anti- and pro-apoptotic proteins, the ratio of which
controls the fate of the cell (Boise et al., 1993;
Oltavai et al., 1993). Structural and biochemical
20   studies of Bcl-2 family members point to a possibility
that these proteins form ion channels (Antonsson et al.,
1997). In addition, Bcl-2 family members bind to
several unrelated proteins including R-ras, Nip-1,-2,-3,
Ced-4-like proteins, Bag-1, Lamin A/C, and p28Bap31.
25   Although the functional significance of some of these
interactions are not yet known, these findings suggest
that Bcl-2 regulates multiple signaling pathways that
influence apoptosis.

There is growing evidence from several independent
30   studies that Bcl-2-related proteins can trigger changes
in gene expression which may or may not be related to
their role in apoptosis regulation (Linette et al.,
1996; Miyashita et al., 1997). Initial studies of E1B

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19K mutant viruses raised the possibility that 19K
functions to dampen transcriptional activation by E1A.
Subsequently, it has been shown that E1B 19K and Bcl-2
alleviate the *trans*-repressive activity of E1A and p53.

5   This derepression of transcription has been correlated
with an up-regulation of one transcriptional target of
p53, Mdm-2 as described in Example I. Since Mdm-2
inhibits p53 transcriptional activity and apoptosis,
this model provides an alternative mechanism by which

10   E1B 19K and Bcl-2 can regulate apoptosis. Furthermore,
Bcl-2 family members may actually control several
additional transcription factors including NF-κB, NFAT
(nuclear factor of activated T cells), c-Jun, and the
glucocorticoid receptor, thus indicating that Bcl-2 and

15   its related proteins can have multiple effects on gene
expression that may contribute to apoptosis.

Compared to apoptosis regulation, relatively little
is known concerning the gene regulatory function of the
Bcl-2 family. In accordance with the present invention,

20   a novel transcriptional repressor, Btf, which
contributes to the modulation of transcription by Bcl-2
related proteins is provided. Btf was identified in a
yeast-two hybrid screen against E1B 19K and subsequently
shown to also interact with Bcl-2 and Bcl-x_L. These Bcl-

25   2 family members inhibit the translocation of Btf to the
nucleus and abrogate its transcriptional activity. The
data presented herein reveal that sustained over-
expression of Btf induces apoptosis and suppresses
transformation by E1A and E1B 19K or mutant p53. Thus,

30   the interaction Btf provides yet another novel pathway
by which the Bcl-2 family can regulate transcription and
control apoptosis.

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Yeast Two Hybrid Assay

　　　In order to identify novel cellular proteins that
interact with E1B 19K, we screened a HeLa cDNA library
using the yeast two-hybrid assay. The library was
5   constructed in the pGAD-GH plasmid in which the cDNA
sequences were fused to the GAL-4 activation domain, and
the bait gene, E1B 19K, was fused to the GAL-4 DNA-
binding domain in the pGBT9 vector (Han et al., 1996).
The plasmids were transformed into the YGH1 yeast strain
10   and screened for GAL-4 inducible phenotypes, namely
growth in the absence of histidine and production of β-
galactosidase. Three million transformants were
screened yielding seven clones (BP-1 to BP-7) that
specifically interacted with 19K and not with the pGBT9
15   vector alone or with an irrelevant protein, Apc-2. BP-
2, -3, and -4 were previously reported as Lamin A/C,
Bax, and Nbk/Bik, respectively. Here, we describe one
of the novel E1B 19K associated proteins, BP-1. A 1.5
kB cDNA containing *bp-1* was isolated seven times during
20   the two-hybrid screen. Since Bcl-2 family members are
highly homologous and frequently interact with the same
cellular proteins, we tested BP-1 for interaction with
Bcl-2-related apoptosis regulators. In addition to
binding E1B 19K, BP-1 interacted with other related
25   proteins, Bcl-2 and Bcl-x_L, but not to the pro-apoptotic
family member, Bax (Fig. 19A).

　　　The interaction between anti- and pro-apoptotic
Bcl-2 family members generally occurs via their
conserved domains, designated Bcl-2 homologous regions
30   1, 2, 3, and 4 (BH1, BH2, BH3, and BH4). BH1-3 are in
close proximity forming a hydrophobic cleft that is
required for dimerization. To determine the regions of
E1B 19K that are required for interaction with BP-1, we

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tested its ability to bind a series of E1B 19K missense and deletion mutants (See Figures 19 A,B & C). These mutants have been characterized previously for binding to other E1B 19K binding proteins, including Bax,

5   Nbk/Bik, Lamin A/C, and Ced-4. Thus, these experiments allow us to compare the binding requirements within the E1B 19K protein against other 19K-associated proteins.

The E1B 19K protein may be divided into three regions: a moderately conserved N-terminus which

10   includes BH3, a highly conserved central region containing BH1, and a poorly conserved C-terminus. It is important to note that most of the deletion mutants tested ΔN30, ΔN64, ΔN87, ΔC93, ΔC70, 30-146, 30-93, and 64-136) failed to interact with BP-1 or with Bax,

15   Nbk/Bik, and Lamin A/C, and that this may be due to abnormal protein folding or masking of the binding site(s) (Fig. 19B). Like Bax, Nbk/Bik, and Lamin A/C, binding of BP-1 was retained in C146 which contains both BH1 and BH3. The small E1B 19K fragment 19-57

20   containing only BH3 was also able to bind BP-1 (Fig. 19B). This fragment is also sufficient for binding to Bax as well as Ced-4. Surprisingly, we found that another small region of E1B 19K, C36, also retained binding to BP-1 (Fig. 19B). This region did not bind to

25   other E1B 19K-associated proteins, Bax, Nbk/Bik, Lamin A/C, or Ced-4. The binding of BP-1 with the E1B 19K mutants C36 and 19-57, suggests that the region of 19K immediately adjacent to BH3 (19-36) may be sufficient for the interaction. This would represent a unique

30   domain that contributes to protein-protein interactions and may correspond to the E1B 19K BH4, although homology is weak.

We further addressed the binding specifications for

BP-1 using several point mutants (pm7, pm51, pm87, and
pm102) that have previously been analyzed for
interaction with E1B 19K-associated proteins as well as
for their ability to inhibit apoptosis. While pm7 and

5   pm102 retained ability to bind to BP-1, substitution of
either phenylalanine for serine at position 51 (pm51) or
glycine for alanine at position 87 (pm87) resulted in a
loss of binding (Fig 19A). The lack of binding with
pm51 is consistent with a role of BH3 in the interaction

10   between E1B 19K and BP-1. Although the loss of binding
with pm87 might suggest a role in BH1, it should be
noted that the glycine residue at position 87 is
absolutely conserved within the Bcl-2 family and is
located in an integral region adjacent to the

15   hydrophobic cleft which serves as the BH3 binding
pocket. Therefore, the pm87 mutant may interfere with
the BH3 region and/or result in a highly misfolded
protein. Indeed, pm87 is generally defective in binding
and at inhibiting apoptosis. Thus, taken together, the

20   mutational analyses indicate that the BH3 and the
adjacent N-terminal sequences (possibly BH4) may play a
more critical role in the interaction with BP-1. This
binding profile overlaps but is distinct from that for
Bax and Nbk/Bik and corresponds to a region of E1B 19K

25   that is required for inhibition of apoptosis.

Characterization of BP-1
        To determine the size and distribution of *bp-1*, we
performed a Northern blot using poly A⁺ RNA prepared from

30   various tissues. Two transcripts were detected at 5 kB
and 3 kB which appeared to be ubiquitously expressed,
although only low levels were detected in the liver
(Fig. 20A). The larger transcript appeared to be

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expressed more abundantly than the shorter form. Since
the largest fragment of *bp-1* obtained in the two-hybrid
screen was only 1.5 kB, we sought to recover full-length
cDNAs corresponding to *bp-1*. Using conventional library

5   screening techniques along with database searches we
were able to identify both full length transcripts which
were named *btf* (Bcl-2-associated transcription factor).
The 3 kB transcript, btf_s, was isolated by screening a
HeLa λ-cDNA library. The 3'-end of btf_s was identical to

10   *bp-1* except that it was missing 147 base pairs (bp)
within the predicted coding sequence. While the 5 kB
transcript, btf_L, could not be recovered from the λ
screen, it was identified as a full-length expressed
sequence tag (EST) within GenBank (accession # D79986)

15   that was isolated from the human KG-1 cell line. Unlike
btf_s, btf_L did contain the 147 bp region present in *bp-1*.
Based on sequence comparisons from GenBank with btf_s, we
found that the remainder of the coding sequences between
btf_s and btf_L were the same, and the large size

20   difference between the transcripts results from
different 3'-untranslated regions (UTRs).

The EST encoding Btf_L was utilized to identify the
sub-chromosomal location of *btf*. However, it remains to
be determined whether btf_L and btf_s are formed from

25   separate genes or whether they are generated by
alternative splicing of the same gene. Using the
UniGene collection assessed through National Center for
Biotechnology Information (NCBI), we found a 137 bp PCR
fragment within the 3'-UTR of the EST (dbSTS entry

30   G20483) that mapped to chromosome 6 between markers
D6S292 and D6S1699. These markers correspond to 6q22-
23, a locus with a high frequency of deletions in
tumors, particularly lymphomas and leukemias (Mitelman,

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1995). Thus, the locus of *btf* correlates with a chromosomal region that may contain a tumor suppressor gene.

To test whether *btf* is actually deleted in tumors, we performed a Northern blot using mRNA prepared from several human cancer cell lines. While both transcripts of *btf* were observed in most cell lines tested, they appeared to be less abundant than normal cells and *btf* was not detected in Raji cells which were derived from Burkitt's lymphoma (Fig. 20B). Indeed, Raji cells were the one cell line tested with known 6q deletions. The Northern blot data was therefore consistent with the chromosomal mapping of the gene. Thus, loss of btf may actually contribute to tumor formation in these cells.

The primary amino acid sequence encoded by the long and short forms of *btf* are compared in Fig. 21. Btf_L is 918 amino acids and has a predicted molecular mass of 106 kDa, whereas Btf_s is missing 49 amino acids near the carboxyl terminus (amino acids 797-846 of Btf_L) and has a predicted size of 101 kDa. See Figures 21 and 22. The original *bp-1* clone encodes for amino acids 384-918 of Btf_L (Btf ΔN384) which is slightly more than half of the full-length protein (Fig. 22). In general, the 49 amino acid region specific to Btf_L and ΔN384 contains highly charged residues (48%), but the significance of this region is not known. Both Btf_s and Btf_L were able to bind E1B 19K in the yeast two-hybrid assay (data not shown). While the overall protein sequence of Btf is not significantly homologous to other known proteins, searches for conserved motifs using the PROSITE database revealed two nuclear localization sequences (NLS) (Fig. 21) as well as putative DNA-binding domains (Fig. 22). There was 88% homology to the basic zipper (bZIP) DNA-

binding domain between amino acids 110 and 126, and 80%
homology to the Myb DNA-binding domain within amino
acids 522 and 531 of Btf (Fig. 22).

5   *Btf$_s$ is a nuclear DNA-binding protein that is sequestered
by the Bcl-2 family*

Since *btf$_s$*, but not *btf$_L$*, was isolated from the HeLa
library, we concentrated our functional assays using the
shorter variant. Btf$_s$ was cloned into the pcDNA3 vector
10   with a Myc epitope for *in vitro* translation and
mammalian expression. The protein sequence of Btf$_s$
suggested that it may bind to DNA. To test this
hypothesis ^{35}[S]-labeled *in vitro* translated Myc-Btf$_s$ was
incubated with native DNA-cellulose. DNA-binding was
15   detected with Btf$_s$ but not with E1B 19K used as a
negative control (Fig. 23). The strength of the
interaction was comparable to a known transcription
factor, Msx-1, and was not competed by RNA (data not
shown). *In vitro* translated E1B 19K and Bcl-2 (data not
20   shown) did not inhibit the ability of Btf$_s$ to bind to DNA
suggesting that the binding sites for DNA and E1B
19K/Bcl-2 are in distinct domains within Btf$_s$. Indeed,
comparison of the putative DNA-binding domains and the
BP-1 (Btf ΔN384) protein which is sufficient for binding
25   to the Bcl-2 family members is consistent with there
being two separate domains.

To confirm the association between Btf$_s$ and Bcl-2
family members we performed an *in vitro* binding assay.
^{35}[S]-labeled *in vitro* translated Myc-Btf$_s$ or Myc-Bax,
30   used as a positive control, was combined with E1B 19K,
Bcl-2, and Bcl-x$_L$. Immunoprecipitation with an antibody
against Myc revealed that Btf$_s$ bound to all three of the
anti-apoptotic Bcl-2 family members, but not to

luciferase used a negative control (Fig. 24). However, the strength of these associations were weaker than those with Bax (Fig. 24). These results support the data obtained from the yeast two-hybrid assay.

5   To examine the biological of Btf$_s$ in vivo, we attempted to express the tagged protein in HeLa cells. While were unable to obtain stable expression of Btf$_s$ even using an inducible promoter, transient transfection did produce low levels of Btf$_s$. Although the levels of

10   Btf$_s$ were not high enough to detect by Western blot analysis of whole cell extracts (data not shown) which would allow us to examine whether Btf$_s$ could co-immunoprecipitate with Bcl-2 family members, we were able to visualize Btf$_s$ by immunofluorescence. Its

15   expression was confined to the nucleus and was present in roughly 4% of the cells (Fig 25). This was consistent with the DNA-binding activity, but not with the established localization of Bcl-2-like proteins which are generally found associated with membrane

20   structures, particularly the mitochondria, endoplasmic reticulum, and nuclear envelope. Unlike other Bcl-2 family members, E1B 19K is not normally present in the mitochondria, but rather predominantly localized to the nuclear envelope. However, since the Bcl-2 family can

25   act by sequestering proteins we checked whether E1B 19K, Bcl-2, and Bcl-x$_L$ could alter the subcellular localization of Btf$_s$. First, E1B 19K and Bcl-2 were co-transfected with Myc-Btf$_s$. The cells were fixed 24 hours post-transfection and then double stained for the

30   Myc epitope present on Btf$_s$ (rhodamine) and either E1B 19K or Bcl-2 (fluorescein). While the percent of Btf$_s$-positive cells was similar to that when it was transfected by itself, the subcellular localization was

altered. Btf$_s$ co-localized with E1B 19K and Bcl-2 within the cytoplasm and nuclear periphery in almost all co-expressing cells (Fig. 25). Subsequent experiments were performed to determine if Bcl-x$_L$ could also sequester

5   Btf$_s$ since these proteins also bound in the yeast two-hybrid assay and *in vitro*. We could not co-stain for Btf$_s$ and Bcl-x$_L$ with available antibodies, so we developed a stable HeLa cell line expressing Bcl-x$_L$ and stained for Btf$_s$ using the antibody against the Myc

10   epitope. While control HeLa cells contained 100% nuclear Btf$_s$ expression, only 7% of the Btf$_s$-positive cells in the HeLa-Bcl-x$_L$ cell line displayed nuclear Btf$_s$ staining and almost all of the Btf$_s$ staining was in the cytoplasm in a pattern similar to Bcl-x$_L$ (Fig. 26). The

15   few cells expressing nuclear Btf$_s$ may be accounted for by the variable levels of Bcl-x$_L$ observed in this cell line and/or an incomplete ability of Bcl-x$_L$ to sequester Btf$_s$. Nevertheless, it is in clear contrast to the parental HeLa cells where the expression of Btf$_s$ was completely

20   nuclear. These results suggest that E1B 19K, Bcl-2, and Bcl-x$_L$ can alter the localization, and therefore apparently, the function, of Btf$_s$.

Btf$_s$ represses transcription which is inhibited by the
25   *Bcl-2 family*

Since Btf$_s$ was shown to bind DNA, we checked whether it could modulate transcription. We first performed a one-hybrid assay in yeast by generating a fusion protein of Btf$_s$ with the GAL-4 DNA-binding domain in the pGBT9

30   vector. If Btf$_s$ contained a *trans*-activation domain one would expect that the adjacent activation and DNA-binding domains would lead to GAL-4-inducible phenotypes in yeast. Growth was not detected in the absence of

histidine suggesting that Btf$_s$ does not contain a transcriptional activation domain (data not shown). However, it remains possible that Btf$_s$ requires mammalian co-factors in order to activate transcription or that

5   Btf$_s$ represses, rather than activates, transcription.

To address these issues, Btf$_s$ was cloned into a mammalian expression vector (pm1) containing the GAL-4 DNA binding domain. Transcriptional activity was monitored using a reporter construct containing GAL-4

10   promoter sites. Transient expression of pm1-Btf$_s$ with the luciferase reporter led to nearly a 10-fold decrease in transcription compared to an empty pm1 vector control (Fig. 27). This effect is comparable to the *trans-repressive* activity of other proteins, such as p53 and

15   Msx-1. Two deletion mutants of Btf$_s$, ΔN522 (amino acids 522-918 without residues 797-846 and ΔC210 (amino acids 1-210) were also cloned into pm1 to determine the general regions that may contribute to transcriptional repression (Fig. 22). Both deletion mutants of Btf$_s$ were

20   detectably expressed in HeLa cells to levels comparable with wild-type Btf$_s$ (data not shown). While Btf$_s$ ΔN552 was not able to repress transcription, the ΔC210 mutant was sufficient for repression and nearly as potent as full-length Btf$_s$ (Fig. 27). Interestingly, this fragment

25   which contains the bZIP homology segment is also rich in serine and glycine residues, a feature that is present in other transcriptional repressors.

Since the Bcl-2-like proteins were capable of sequestering Btf$_s$ in the cytoplasm, we hypothesized that

30   they may also block the ability of Btf$_s$ to repress transcription. To test this possibility we co-transfected E1B 19K, Bcl-2, and Bcl-x$_L$ with pm1-Btf$_s$ and the luciferase reporter construct. In the control

samples, none of the Bcl-2 family of proteins had a significant effect on transcription of the luciferase reporter (Fig. 27). However, transfection of any of the three Bcl-2 family members (E1B 19K, Bcl-2, and Bcl-x_L),

5   but not the empty pcDNA3 vector, abrogated Btf$_s$-mediated transcriptional repression. These data suggest that E1B 19K, Bcl-2, and Bcl-x_L inhibit Btf$_s$ *trans*-repression, probably by binding to and sequestering Btf$_s$ in the cytoplasm.

10

Sustained expression of Btf$_s$ inhibits transformation

Transfection of the adenovirus *E1A* gene along with *E1B 19K* or *bcl-2* in primary BRK cells induces transformation as a consequence of dual proliferative

15   and anti-apoptotic signaling. Expression of E1B 19K/Bcl-2 binding proteins, such as Bax and Nbk/Bik, antagonizes the anti-apoptotic signal and causes a reduction in foci formation. To determine if Btf$_s$ could also antagonize the ability of E1B 19K to transform

20   primary cells, we transfected E1A and E1B 19K, with and without Myc-Btf$_s$, into primary BRK cells. Btf$_s$ caused a 64% reduction in foci formation mediated by E1A and E1B 19K (Fig. 28A). This suggests that interaction between Btf$_s$ and E1B 19K inhibits 19K function *in vivo* and/or

25   Btf$_s$ is capable of suppressing transformation independent of 19K. The reduction in foci formation by Btf$_s$ and E1A compared to E1A alone suggested the later is possible (Fig. 28A). However, we tested whether Btf$_s$ could inhibit another transforming signal, E1A with p53DD.

30   p53DD is a p53 deletion mutant that contains the C-terminal oligomerization domain and functions in a dominant-negative fashion to inhibit p53-mediated apoptosis and growth arrest. Since p53DD blocks both of

these processes, it produces a very potent transforming signal. Here we show that expression of Btf$_s$ was capable of repressing foci formation by E1A and p53DD by about 60%, suggesting that btf$_s$ may act as a general suppressor of transformation (Fig. 28B).

Btf$_s$ functions to induce apoptosis

To further characterize the transformation suppressing activity of Btf$_s$ we tested the effect of its expression on apoptosis and cell cycle progression. Attempts to generate stable BRK or HeLa cell lines expressing Btf$_s$ failed, indicating that sustained Btf$_s$ expression is incompatible with either cell proliferation or viability. To monitor Btf$_s$ expression in transient assays, the cDNA was cloned into the pIRES-EGFP vector which provides co-expression of Btf$_s$ and the enhanced green fluorescent protein (EGFP) marker. HeLa cells were transiently transfected and the cells were harvested 48 and 72 hours post-transfection. The cell cycle kinetics were analyzed following propidium iodide staining by FACS analysis. Since EGFP staining with Btf$_s$ was not detectable until 48 hours post-transfection, we were unable to characterize the cell cycle characteristics at the earlier time points. The levels of EGFP expression and the cell cycle kinetics at 48 and 72 hours are shown in Table 1. At 48 hours post-transfection there were only 19.5% EGFP-positive cells in the presence of Btf$_s$, whereas there were 61.2% positive cells in the control pIRES-EGFP empty vector. The FACS analysis demonstrated that Btf$_s$ led to an increase in percent of subG$_{0/1}$ cells from 3.4% to 15.8% indicating an increase in cell death. No other obvious changes in cell cycle parameters were observed at this

time point.

　　　To test whether E1B 19K could inhibit this cell death, we co-transfected pIRES-EGFP-Btf$_s$ with pCMV-E1B 19K. E1B 19K inhibited Btf$_s$-mediated cell death at 48 hours as indicated by an increase in the number of EGFP expressing cells from 19.5% to 34.3% and fewer subG$_{0/1}$ cells (15.8% to 10.1%) (Table 1). Thus, E1B 19K expression abrogated cell death induced by Btf$_s$. At 72 post-transfection the amount of cell death triggered by Btf$_s$ increased and could no longer be inhibited by E1B

TABLE 1. Overexpression of Btf_s induces cell death[a]

Vector	48 h posttransfection					72 h posttransfection				
	% EGFP positive	% sub-$G_{0/1}$	% $G_{0/1}$	% S	% G_2/M	%EGFP positive	% sub-$G_{0/1}$	% $G_{0/1}$	% S	% G_2/M
pIRES-EGFP + pcDNA3	61.2	3.4	42.3	20.0	33.7	45.7	5.6	32.5	20.9	40.6
pIRES-EGFP + pCMV-E1B 19K	72.0	1.5	54.2	21.0	23.6	59.0	8.8	55.4	20.8	14.8
pIRES-EGFP-Btf_s + pcDNA3	19.5	15.8	36.7	21.7	25.6	13.2	29.1	33.2	20.6	16.7
pIRES-EGFP-Btf_s + pCMV-E1B 19K	34.3	10.1	41.8	17.8	30.0	15.4	36.9	36.0	13.3	13.6

[a] HeLa cells were electroporated with 20 μg of pIRES-EGFP-Btf_s or empty vector in combination with 10 μg of pCMV-E1B 19K or empty pcDNA3 vector. The cells were harvested 48 and 72 h posttransfection, fixed in 2% paraformaldehye, and stained with PBS containing 1 μg of propidium iodide per ml. 250 μg of RASE A per ml. and 0.1% Tween 20. The cells were incubated for at least 30 min at room temperature and then analyzed by FACS. The results show the percentage of EGFP-positive cells and the cell cycle kinetics for the EGFP-positive population.

19K. In the presence of Btf_s there were only 13.2% EGFP positive-cells, 29.1% of which were represented in the $subG_{0/1}$ peak (Fig. 29). The 72 hour time point also revealed a decrease in the G_2/M peak with Btf_s (16.7%) compared to the control vector (40.6%). The change in G_2/M by Btf_s may be an indication that cells are exiting from these phases of the cell cycle to go into apoptosis.

Interestingly, we also observed a decrease in the G_2/M peak with E1B 19K in the absence of Btf_s. At 72 hours post-transfection there was a change in the number of cells in G_2/M from 40.6% with the control vector to 14.8% in E1B 19K transfected cells. Changes in cell cycle progression by the anti-apoptotic Bcl-2 family members have been observed previously, such that generally they produce a decrease in cell cycle progression. While others have shown that Bcl-2 causes an increase in $G_{0/1}$, here we show that E1B 19K causes an decrease in G_2/M. Thus, these results may reflect a difference in the mechanism between the cell cycle regulation by E1B 19K and other related proteins.

Cell death has been classified as either necrosis or apoptosis, where necrosis is considered a passive process of cell death associated with trauma, and apoptosis is a genetically programmed active response leading to cell suicide. Unlike necrosis, apoptosis involves morphological changes such as chromatin condensation, DNA fragmentation, and cytoplasmic blebbing. To test whether the increase in $subG_{0/1}$ cells by Btf_s was due to apoptosis, we examined the nuclei of EGFP-positive cells with Hoechst dye 72 hours post-transfection. Chromatin condensation observed by this approach would be one indication of apoptosis. HeLa

cells transfected with the empty pIRES-EGFP vector
showed EGFP stained cells with normal nuclei. However,
in cells transfected with pIRES-EGFP-Btf$_s$, the EGFP-
positive cells had condensed chromatin staining,

5   indicating that they were dying by apoptosis (Fig. 30
Taken together, these results suggest that Btf$_s$ can
function to promote apoptosis which may account for its
ability to suppress transformation. E1B 19K can inhibit
Btf$_s$-induced apoptosis, albeit incompletely in some

10   assays. None the less, this suggests that the Bcl-2
family affects apoptosis through modulation of
transcription.

DISCUSSION

15   The Bcl-2 family regulates apoptosis through
multiple mechanisms. For example, these proteins can
function at the mitochondria by possibly forming
channels that regulate mitochondrial membrane potential
and cytochrome C release. Alternatively, the Bcl-2

20   family may directly regulate caspase activation through
interactions with Ced-4-like proteins. We describe a
novel E1B 19K-interacting protein, Btf, isolated through
a two-hybrid screen that regulates an alternative
pathway to control apoptosis. Two transcripts

25   corresponding to *btf* were identified, *btf*$_s$ and *btf*$_L$.
Both appeared to be widely expressed, but were deleted
in some tumors. Here, we described the function and
biological significance of the protein product generated
from the shorter form, Btf$_s$, which differs from Btf$_L$ in

30   just a 49 amino acids in the C-terminal region.
Double staining experiments were able to show the
co-localization of Btf$_s$ with E1B 19K, Bcl-2 and Bcl-x$_L$,
thereby supporting the interactions observed in the

yeast two-hybrid assay as well as by *in vitro* co-immunoprecipitation. While transiently expressed Btf_s was nuclear and could be sequestered into the cytoplasm by the anti-apoptotic Bcl-2 family members, the

5   localization of endogenous Btf_s remains to be determined. It is entirely possible that in normal, non-apoptotic cells Btf_s is predominantly expressed in the cytoplasm. In contrast, in dying cells, where there is a higher percentage of pro-apoptotic versus anti-apoptotic Bcl-2

10 family members, Btf_s may translocate to the nucleus and potentiate apoptosis.

Btf_s was able to bind DNA *in vitro*, and in reporter assays Btf_s repressed transcription. The *trans*-repressive activity was inhibited by E1B 19K, Bcl-2 and

15 $Bcl-x_L$ correlating with their cytoplasmic sequestration potentials. While it remains possible that part of this phenomenon is a result of Btf_s-induced apoptosis, the amount of cell death observed at 24 hours would not account for the 10-fold reduction in transcriptional

20 activity. The evidence that Btf_s is a transcription repressor was further supported by the identification of the ΔC210 deletion mutant that was sufficient for repressing transcription. The N-terminal fragment of Btf_s is rich in glycine and serine, a feature that is

25 common to transcriptional repressors. Taken together, these data provide evidence for a novel *trans*-repressive protein that triggers apoptosis which is sequestered and inhibited by Bcl-2 family members.

One of the features of the Bcl-2 family exemplified

30 by the Btf_s interaction is their ability to sequester other cellular proteins from their normal subcellular localization. This process enables E1B 19K and Bcl-2 to inhibit apoptosis using more than one cellular pathway.

The best characterized example concerns the association between pro-apoptotic and anti-apoptotic Bcl-2 family members. While these interactions have been known for several years, recent studies of E1B 19K and Bax have demonstrated that these family members can alter each other's sub-cellular localization. Bax is normally stimulated to go to the mitochondria during apoptosis and causes a loss in mitochondrial membrane potential. However, over-expression of E1B 19K causes Bax to be sequestered to the nuclear periphery where E1B 19K is localized. This process most likely blocks the ability of Bax to disrupt mitochondrial function. The Bcl-2 family also associates with several unrelated proteins that may contribute to apoptosis regulation. For example, Bcl-2 associates with and targets the serine/threonine kinase Raf-1 to the mitochondria. Over-expression of Bcl-2 and Raf-1 cooperate to inhibit apoptosis. Another example that is now emerging is the association between the anti-apoptotic Bcl-2 family members with *C. elegans*, Ced-4, and with its mammalian homologue Apaf-1. These interactions directly block the activation of downstream caspases. Thus far, the *C. elegans* Bcl-2 homologue, Ced-9, as well as Bcl-x_L and E1B 19K have been shown to bind to Ced-4, and at least Ced-9 and E1B 19K can redistribute Ced-4 from the cytosol to cytoplasmic membranes. Bcl-x_L also interacts with Apaf-1 and therefore it will be interesting to determine whether Bcl-x_L can alter the localization of Apaf-1. In perhaps an analogous scenario, E1B 19K also sequesters the death-promoting protein FADD, an upstream component of Fas- and TNF-α-mediated death signaling pathway. Overexpressed FADD becomes multimerized and produces filaments throughout the cell. E1B 19K disrupts the

FADD filaments causing FADD to relocalize with regions normally associated with 19K and inhibits FADD-dependent apoptosis. Here, we show that the Bcl-2 family members also sequester a nuclear transcription factor and that this may also play a role in apoptosis. The minimal region for E1B 19K required for interaction with Btf at the amino terminus (amino acids 1-36) appears to be distinct from the interaction regions involved in binding to other pro-apoptotic proteins (Bax, Ced-4, Nbk/Bik). This amino terminal region of E1B 19K may correspond to BH4 of Bcl-2 and Bcl-x_L, although this remains to be tested directly. Together these studies suggest that E1B 19K and possibly other Bcl-2 family members, act as binding proteins for a number of apoptosis regulators which could contribute to their widespread role as apoptosis inhibitors.

Transcriptional regulation often plays a critical role during apoptosis by either activating or repressing genes encoding basic apoptotic components. Indeed, inhibition of RNA and protein synthesis block apoptosis induced by a number of circumstances, including growth factor deprivation and following treatment with some chemotherapeutic drugs. In contrast, others have shown that these inhibitors can actually promote cell death, suggesting that loss of a short-lived survival factor can also lead to apoptosis. A number of transcription factors have been identified that may serve as positive or negative regulators of apoptosis. For example, the NF-κB transcription factor plays an important role in blocking apoptosis triggered by TNF-α although the target genes for NF-κB are not yet known. However, in other situations, NF-κB activation has been associated with induction of apoptosis. Furthermore, Bcl-2 and E1B

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19K represses NF-κB activity providing another mechanism for Bcl-2 family members to control apoptosis.

Another transcription factor that may be regulated by the Bcl-2 family during apoptosis is p53. The p53 tumor suppressor protein is required for apoptosis during ionizing radiation and chemotherapeutic drugs, as well as transforming oncogenes such as *c-myc* and *E1A*. While p53 may have multiple functions, the transcriptional activity of p53 is clearly critical for the regulation of cell death in some situations but not all. However, it is still unclear whether p53-mediated apoptosis requires its transcriptional activation or repression properties, or perhaps both. On the one hand, p53 *trans*-activates both *bax* and fas, both of which are bonafide inducers of apoptosis. In contrast, several p53-repressible genes have been identified which may potentially contribute toward apoptosis, including bcl-2, MAP4, interleukin-6, c-fos, and c-myc. Expression of Bcl-2 and E1B 19K alleviate the transcriptional repression activity of p53 providing a mechanism for Bcl-2 family regulation of apoptosis. Thus, Bcl-2 family members can influence apoptosis by modulating the transcriptional activity of both NF-B and p53. Inhibition of Btf_s-mediated transcriptional repression and apoptosis by the Bcl-2 family establishes another connection between these apoptosis regulators and modulation of transcription. It will certainly be interesting to determine whether Btf_s can influence the activities of other transcription factors related to apoptosis, such as NF-κB and p53.

We would predict that Btf_s may repress the transcription of survival genes. Based on previous observations of regulation of gene expression by E1B

19K, one possible target may be the p53 inhibitor, Mdm-2. We have previously observed that stable expression of E1B 19K or Bcl-2 leads to an increase in Mdm-2 mRNA and protein levels. Such a mechanism could also account for the ability of E1B 19K to derepress the transcriptional repression mediated by p53 and E1A. While it will be worthwhile to determine whether Btf_s could affect p53 modulation of these targets, the fact that Btf_s was capable of inhibiting transformation by p53DD suggests that another target also exists that is independent of p53. We also have not been able to detect binding between Btf_s and p53 (data not shown), although it is still plausible that Btf_s associates with p53-dependent co-activators such as p300. Since the putative DNA-binding sites within Btf_s contains homology to bZIP and Myb, it is conceivable that they share similar targets. Interestingly, correlations exist between both of these transcription factor families with apoptotic regulation. For example, application of functional blocking antibodies against the AP-1 proteins, Fos and Jun, or transfection with dominant-interfering Jun, inhibits apoptosis in neuronal cells following growth factor withdrawal. However, relevant target genes that contribute toward AP-1-mediated apoptosis have yet to be identified. The Myb family of transcriptional regulators inhibit apoptosis, and regulate the transcriptional activation of bcl-2. This may therefore provide a feedback loop between Btf_s and Bcl-2. Future studies will be performed to determine whether Btf_s regulates *bcl-2*, or other genes involved in apoptosis. The link between Btf_s and the Bcl-2 family may provide an alternative mechanism by which the Bcl-2 family is able to regulate cell survival.

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While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.